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(54) Title: A SOLUBLE BETA 2 MICROGLOBULIN (β2M)/HFE MONOCHAIN FOR BIOTECHNOLOGICAL AND THERAPEUTIC APPLICATIONS

(57) Abstract: A DNA construct encodes a monochain form of beta-2 microglobulin/HFE protein. Particularly, a genetically engineered DNA construct encompasses human beta-2 microglobulin (β2m) gene linked via an adjustable linker to specific domains of the human HFE gene, in order to produce a properly conformed, soluble, stable and functional protein. The construct may be used as a platform for drug delivery of the therapeutics of cancer, autoimmune diseases, inflammatory conditions and the like. It can also be used for the therapeutics of iron-overload conditions, oxidative stress conditions and chronic infections.

A SOLUBLE BETA 2 MICROGLOBULIN (β2M)/HFE MONOCHAIN FOR BIOTECHNOLOGICAL AND THERAPEUTIC APPLICATIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of U.S. provisional application 60/234,843, filed September 22, 2000, the entire contents of which being hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

[0002] The present invention relates to methods for targeting and delivering of drugs into human cells including without limitation, tumors and metastasizing cells, activated lymphocytes operative in autoimmune and inflammatory conditions, and the like. This invention also relates to therapeutic interventions for iron overload disorders and chronic infections.

Background of the Technology

[0003] Millions of people suffer from cancer and autoimmune diseases all over the world. Currently, most cancer and some autoimmune diseases remain incurable, and treatments provide the means to prolong life expectancy and enhance qualities of life of the sick but do not provide remedy. Current therapies are facing major problems, particularly:

1. Lack of specificity. Chemotherapy, radiotherapy and other anti-cancer therapies damage healthy tissues and consequently, induce severe side effects.

2. Development of resistance to anti-cancer therapies and specifically, to chemotherapy, a phenomena known as the multi-drug resistance.

[0004] One approach to attack these problems is to target and modulate the major cellular iron (Fe) transport system, the transferrin receptor (TfR). Since iron is indispensable for cellular proliferation, it is excessively uptaken, via the TfR,

by tumors, metastases and activated autoimmune lymphocytes as well as lymphocytes and leukocytes active in inflammatory disorders and graft rejection (Ragheb et al 1999, Bayer et al, 1999).

[0005] Previous approaches that manipulated the cellular iron uptake system for cancer therapy included the following:

1. Iron supply to tumor cells may be blocked, thus, leading to growth arrest and death (Kemp, 1997). This was achieved by utilizing gallium nitrate, a chemical structurally resembling iron that competes with intracellular iron uptake (Apseloff et al, 1997, Collery et al, 2000, Webster et al, 2000). However, this chemical is not specific for TfR and therefore interferes with various iron uptake systems in the body and causes severe side effects.

2. Drugs may be delivered into cells through the internalization of the TfR (Singh, 1999, Hagihara et al, 2000, Hall, 2000). This was achieved by conjugation of drugs to transferrin, the natural ligand of TfR. Although transferrin conjugated to doxorubicin could overcome multi drug resistance in a cancer cell line (Wang et al, 2000), the administration of transferrin-conjugates is not an optimal approach since transferrin stimulates growth, which is contradictory to the purpose of the treatment. Moreover, it is diluted in the blood due to high levels of serum transferrin.

3. Anti-TfR antibodies may be used for drug delivery into cells (Taetle et al, 1987, White, et al, 1990, Kemp et al, 1992, Brooks et al, 1995, Wening et al, 1999). Usage of these antibodies has some drawbacks since they do not dissociate from the receptor inside the cells but recycle back to the cell surface. Thus, the drug-conjugated antibodies may exert a cytotoxic effect on surrounding cells and tissues. In addition, the antibodies developed thus far provoke immune responses, thus, preventing multiple treatments.

[0006] HFE is a central regulator of iron absorption from the diet and iron uptake by most cells in the body. A recessive autosomal mutation (C282Y) in the HFE gene causes an iron overload disorder (Hereditary Hemochromatosis, HH) common in populations of Northwest European ancestry (Feder et al, 1996; Beutler et al, 1996; Jazwinska et al, 1996; Jouanolle et al, 1996; Barton et al, 1997; Borot et al, 1997; Carella et al, 1997; Worwood, 1997). HH patients accumulate iron first in the transferrin pool and later in parenchymal tissue of many organs leading to multi-organ dysfunction including diverse pathologies of the liver (cirrhosis), pancreas (diabetes), joints (arthritis), heart (arrhythmia), and hypophysis (hypogonadism). Liver damage, including hepatocellular carcinoma, is the major cause of mortality in HH (Niederau et al, 1999).

[0007] Positional cloning of the HFE gene from an HLA-linked region of chromosome 6p (detailed in a review by Feder, 1999) and the subsequent crystallization of the encoded protein (Lebron et al, 1998) revealed significant sequence and structural homology to MHC class I proteins (37% identity in the ectodomain). The gene encodes a 348-residue type I transmembrane glycoprotein, which associates with the class I light chain β2m, and is mutated in most patients with HH (Feder et al, 1996; and U.S. patent 6,140,305). In both HFE and classical class I molecules, the α1-α2 superdomain forms a platform comprising an eight strand antiparallel β-sheet topped by two helices, which is positioned on top of immunoglobulin constant-like domain α3 and β2m. A mutation in the HFE gene is the primary risk factor for the HH disease. 75-90% of affected individuals with clinical HH are homozygous for a position C282Y mutation (Feder et al, 1996). This mutation prevents the formation of a critical disulfide bond in the HFE α3 domain, impairing its ability to form its obligate heterodimer with β2m and, thus, inhibits its delivery to the cell surface (Feder et

al, 1998). Mice deficient in β2m and mice deficient in HFE or mice engineered to express the disease-associated C282Y HFE mutation demonstrate systemic iron overload with iron deposition in liver parenchymal cells (de Sousa et al, 1994, Rothenberg et al, 1996, Zhou et al, 1998, Bahram et al, 1999, Levy et al, 2000). Since it was demonstrated that β2m heterodimerizes with HFE and mice lacking β2m develop iron overload, it was suggested that HFE requires β2m to exert its regulatory effect on iron metabolism. Moreover, it was demonstrated that mice lacking both the HFE and the β2m genes develop more severe iron overload symptoms than mice lacking HFE alone, suggesting that there may be another, still unidentified, molecule that interacts with β2m and regulates intestinal iron absorption. Thus, β2m may play an active role in iron regulation (Levy et al, 2000).

[0008] The interaction of HFE with TfR was demonstrated biochemically by co-immunoprecipitation of HFE with TfR in intestinal cells, placental cells, and HFE-transfected cells (Parkkila et al, 1997; Feder et al, 1998; Gross et al, 1998; Waheed et al, 1999; Salter-Cid et al, 1999). The results were further supported by the co-localization of HFE and TfR on the cell surface and in intracellular organelles (Gross et al, 1998; Feder et al, 1998).

[0009] HFE binds TfR tightly at the pH of the cell surface, but not at pH 6, suggesting that HFE is internalized with TfR and dissociates from the receptor in acidified endosomes (Lebron et al, 1998; Lebron et al, 1999). Biochemical studies showed that HFE and ferrum bound-transferrin (Fe-Tf) can bind simultaneously to TfR to form a ternary complex consisting of one Fe-Tf and one HFE bound to a TfR homodimer (Lebron et al, 1999), and that HFE inhibits the TfR-Tf interaction (Feder et al, 1998; Gross et al, 1998) by binding near or at the Tf-binding site of TfR (Lebron et al, 1999). The crystal structure of a complex between the ectodomains of HFE and TfR

(Bennet et al, 2000) allowed the localization of the TfR binding site on HFE to the C-terminal portion of the α1 domain helix and an adjacent loop, a region distinct from the ligand-binding site of a classical class I MHC molecule (Lebron et al, 1999).

[0010] HFE-overexpressing cells demonstrate an iron-deficiency phenotype; they have decreased ferritin expression (Gross et al, 1998; Roy et al, 1999; Corsi et al, 1999), modestly increased number or TfRs (Roy et al, 1999), and induced IRP levels (Corsi et al, 1999; Riedel et al, 1999), all implying low intracellular iron pools.

[0011] Immunohistochemical analyses demonstrated co-localization of HFE and TfR in intracellular compartments and on the cell surface (Gross et al, 1998; Feder et al, 1998). Further studies of HFE expression in HFE-GFP-transfected hepatoma cell line, showed that HFE co-localizes with TfR mainly in perinuclear regions (Ikuta et al, 2000). The compiled data suggest that TfR-bound HFE is internalized with the receptor and is released within the endosomes.

[0012] Several research groups have engaged the problem of growth arrest and cell death in HFE-transfected HeLa cells. This problem could be solved only when the HFE gene was expressed under the control of an inducible expression system (Roy et al 2000, Riedel et al, 1999).

SUMMARY OF THE INVENTION

[0013] Since β2m appears to play an active role in iron regulation and since proper folding is crucial for function of all MHC class I molecules, including HFE, the present inventors have designed a chimeric monochain protein that maximizes the chances of an HFE-derived polypeptide to associate with its natural counterpart(s) and to gain full conformation and function. This was achieved by linking the two polypeptides

(β2m and specific domains of HFE) via an adjustable linker that brings β2m and HFE-derived polypeptides to appropriate folding.

[0014] The monochain is soluble, stable in serum, and functional, as assessed by its binding to TfR. Consequently, it is optimal for the dual designed functions: (a) drug delivery into target cells, and (b) regulation of intracellular iron uptake.

[0015] In particular, this monochain manifests specific characteristics that are advantageous for drug delivery systems:

1. It is a soluble, stable and fully conformed protein.

2. It binds specifically to TfR and therefore targets cells that over-express this receptor.

3. It is continuously internalized by the target cells, thus enabling efficient drug delivery.

4. It dissociates from the receptor in the cells, thus minimizing side effects.

5. It negatively regulates iron absorption, thus reducing growth of undesired cells and preventing lymphocyte activation.

6. It is not diluted in the blood as is transferrin.

7. It should not induce immune responses since it is a self non-polymorphic protein.

8. Delivery of drugs via this monochain is expected to overcome drug-resistance since it is a natural TfR-binding protein.

[0016] The monochain will serve as a core for the construction of a drug delivery platform where various chemicals, proteins, peptides, DNA or oligonucleotides are conjugated directly to the protein or indirectly via liposomes or polymer-modified liposomes, as has been previously shown for other TfR-targeting vehicles (Zhang et al, 2001, Kono et al, 2001, Bickel et al, 2001).

[0017] Of particular interest is the option of drug delivery to the brain since the blood-brain barrier (BBB) prevents the penetration of most drugs. An exception is the transferrin molecule that, following its binding to TfR on epithelial cells of the BBB, is internalized and reaches brain cells (Bickel et al, 2001). Therefore, any delivery system utilizing the TfR as a target, shares this advantageous property that is crucial in situations of brain metastases, particularly following the development of drug resistance as in the case of Doxil®, a novel form of doxorubicin in polyethylene-glycol coated liposomes (Lyass, et al, 2000). Therapy that combines chemotherapy and TfR-targeting by the monochain is expected to overcome drug resistance and to enable penetration through the BBB, providing a better and more efficient therapeutic solution for advanced stages of cancer.

[0018] This platform may be expanded into a larger three-polypeptide construct that will deliver a variety of proteins, including regulatory components such as anti-oncogenes for the intervention with cancer progression, and proteins that are mutated in hereditary diseases such as the glucocerebrosidase, which is currently administrated to Gaucher patients.

[0019] Since TfR is over-expressed in activated lymphocytes, a monochain-based drug delivery system should be used in cases that involve activated lymphocytes and leukocytes including graft rejection, skin inflammation, autoimmune disorders and the like.

[0020] The monochain molecule could be also utilized as a biotechnological tool for the insertion of desired DNA or proteins into cells. For example, poor transfection efficiency is the major drawback of lipofection (Yanagihara et al, 2000). The addition of transferrin to liposomes enhances the delivery of DNA, oligonucleotides, and peptides into the cells, *in vitro* and *in vivo* (Ogris et al 1999). It is conceivable that the addition of the monochain or its derivatives to liposomes will

improve their intracellular delivery and may serve as a possible strategy for gene therapy.

[0021] Since the monochain reaches the endosomes following its internalization and endosomes serve as a major site for protein cleavage and production of peptides for antigen presentation to regulatory lymphocytes (T helper cells), the monochain can be used as a delivery vehicle for antigens into cells, resulting in the desired antigen presentation to lymphocytes (Ali et al, 1999, Fernandes et al, 2000). In this way, one could enhance and amplify immune responses and improve vaccination strategies.

[0022] Soluble β2m/HFE monochain protein provides a therapeutic solution for treatment of various iron overload conditions, including, without limitation, hereditary hemochromatosis, and secondary iron overload disorders such as transfusions, thalassemias, hemolytic anemias and chronic infections. The β2m/HFE monochain and derivatives also provide a therapeutic solution to oxidative stress disorders resulting in tissue damage, including, without limitation, vascular diseases, inflammation, atherosclerosis, lung injury, ischemia and the like.

[0023] Monochain based therapeutics are to be administered externally as an ointment, internally directly into the circulation in perfusion, or orally as tablets, depending on the drug delivered and the disease.

[0024] Thus, according to the present invention, a DNA construct encoding a soluble monochain β2m/HFE chimera is provided. The expressed protein serves for delivery of therapeutic agents into cells by administering an effective dose of monochain conjugated to a therapeutic agent. The expressed monochain also serves for the regulation of iron absorption and metabolism in target cells.

[0025] The present invention is also directed to the use of HFE alone, or an analog, fragment, functional derivative or

salt thereof, for the treatment of iron overload diseases, such as hereditary hemochromatosis (HH), as well as secondary iron overload diseases, such as transfusions, thalassemias, hemolytic anemias, oxidative stress conditions and chronic infections.

[0026] The present invention further relates to the use of β2m *per se*, its analogs, active fragments, functional derivatives and salts for the treatment of iron overload conditions.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027] Figures 1a and 1b are radiolabeled nitrocellulose membrane blots showing that human tumor cell lines express high levels of TfR and low levels of HFE. Fig. 1c is a table summarizing the results. Cytoplasmic RNA was extracted from human tumor cell lines, fractionated on agarose gel, blotted on nitrocellulose membrane and hybridized with the relevant P^{32}-labeled probes. The following radiolabeled fragments were used: TfR cDNA, HFE cDNA and H-$2K^b$ cDNA. The membrane was exposed for 4h to X-ray film following hybridization with TfR and for 2 weeks following hybridization with HFE probes. The membranes thus obtained are shown in Figs. 1a and 1b. The signals were scanned by phosphorimager, quantitated and the relative strength is summarized in the table of Fig. 1c.

[0028] Figs 1d-1k are graphs showing that human tumor cell lines express high levels of surface TfR. Tumor cell lines of lymphoid origin (K562 (Fig. 1d), CRHF (Fig. 1e), Dami (Fig. 1f), MegO1 (Fig. 1g)) breast carcinoma (T47D (Fig. 1h)) cervical carcinoma (SiHa (Fig. 1i)), and melanoma (GTBS (Fig. 1j), WUBI (Fig. 1k)) were analyzed by FACS for the expression of surface TfR using mAb V1-10. Mean fluorescence values of each cell line are included above each graph.

[0029] Fig. 2 is the DNA (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequence of the β2m/HFE monochain with a C-terminal

myc-tag, followed by a His-tag. The DNA sequence analysis was performed in the sequencing facilities of Tel Aviv University. Amino acid translation was performed by the DNA Strider program.

[0030] Fig. 3 is a membrane showing that HEK293/β2m/HFE/Mhm cells express and secrete β2m/HFE monochain. Individual HEK293/β2m/HFE/Mhm clones M1-M2.5 were tested for expression and secretion of β2m/HFE monochain. Cell extracts and conditioned media were harvested, fractionated on SDS-PAGE, blotted onto nitrocellulose membrane, hybridized with anti-myc mAb and appropriate horseradish peroxidase-conjugated secondary antibodies. Immune detection was performed using an ECL kit according to the manufacturer's directions (Amersham Pharmacia Biotech).

[0031] Fig. 4 is a gel showing that β2m/HFE monochain binds TfR. HEK293 cells, HEK293/HFE, HEK293/β2m/HFE/Mh and HEK293/β2m/HFE/Mhm cells were metabolically labeled for 1h. The cells were harvested and cell extracts were immunoprecipitated using anti-HFE antibody, 2F5, anti-hβ2m antibody, B2.62.2, and anti-hTFR antibody, V1-10 as indicated. The immunoprecipitates were fractionated on SDS-PAGE. The gel was mounted, dried and exposed to X-ray film. Proteins corresponding to 90 kD (TfR), 55 kD (monochain) 45 kD (HFE and/or class I MHC) and 12 kD (β2m) are marked with arrows.

[0032] Figs. 5a and 5b are gels showing synthesis and intracellular trafficking studies of HFE and β2m/HFE proteins. In Fig. 5a, HEK293/β2m/HFE/Mhm cells were metabolically labeled for 30 minutes and chased as indicated. The cells and conditioned media were harvested. Cell extracts were immunoprecipitated using anti-HFE antibody, 2F5, and anti-hTfR antibody, V1-10. The immunoprecipitates and the media (sup.) were fractionated on SDS-PAGE. The gel was mounted, dried and exposed to X-ray film. In Fig. 5b, HEK293/HFE cells were metabolically labeled for 30 minutes and chased as indicated.

Cells were harvested and cell extracts were immunoprecipitated using anti-TfR antibody, V1-10. The immunoprecipitates were fractionated on SDS-PAGE. The gel was mounted, dried and exposed to X-ray film. Proteins corresponding to 90 kD (TfR), and 45 kD (HFE) are marked with arrows.

[0033] Figs. 6a and 6b are gels showing that HeLa cells cannot over-express HFE-derived molecules. In Fig. 6a, HeLa, HeLa/HFE, HeLa/HFE(mut.C282Y) and HeLa/β2m/HFE/Mhm cell lines were tested by RT-PCR for the expression of the transfected genes, using primers corresponding to the α1-α2 domains of HFE and actin, respectively. Non-specific bands are marked with stars. In Fig. 6b, HeLa/HFE and HeLa/β2m/HFE/Mhm cell lines were metabolically labeled for 1 hour, lysed and immunoprecipitated with anti-HFE antibody, 2F5 and anti-hTFR antibody, V1-10. The immunoprecipitates were separated on SDS-PAGE. The gel was mounted, dried and exposed to X-ray films. Proteins corresponding to 55 kD (β2m/HFE monochain) are marked with arrows. Non-specific bands are marked with stars.

[0034] Figs. 7a and 7b are gels showing that radioactive labeled β2m/HFE/Mhm monochain binds TfR and penetrates into cells. Human tumor cell lines (HeLa and FO1) and mouse cell lines (VAD12.79, VAD12.79/hTfR, B16) were incubated, with ^{35}[S]-labeled β2m/HFE/Mhm conditioned media. The cells were harvested and cell extracts were immunoprecipitated with anti-hTfR antibody V1-10, (Fig. 7a) and anti-HFE antibody 2F5 (Fig. 7b) as indicated. The precipitates were fractionated on SDS-PAGE. The gel was mounted, dried and exposed to X-ray film.

[0035] Fig. 8 is a gel showing purification of β2m/HFE/Mhm from conditioned media. β2m/HFE/Mhm proteins were purified as described in the "Specific Techniques" section. Proteins from different purification steps were fractionated onto SDS-PAGE. The gel was divided: half was stained with Coomassie for the estimation of protein concentration (Fig. 8, upper part) and half was blotted to nitrocellulose membrane and hybridized with

anti-myc antibodies followed by horseradish peroxidase-conjugated secondary antibodies (Fig. 8, lower part). Immune detection was performed using an ECL kit according to the manufacturer's directions (Amersham Pharmacia Biotech). S-starting material, FT-flowthrough, W-wash, E-elution. Non-specific bands are marked with stars.

[0036] Fig. 9 shows membranes demonstrating that externally added β2m/HFE/Mhm reduces cellular TfR levels. HeLa cells were incubated for 24 hours with 20 µg/ml purified β2m/HFE/Mhm or treated with 100 µM DFO. The cells were harvested and cell extracts were fractionated on SDS-PAGE, blotted to nitrocellulose membrane and hybridized with anti-hTFR antibody, V1-10 (upper gel), and anti MAP-kinase antibodies (lower gel) followed by the appropriate horseradish peroxidase-conjugated secondary antibodies. Immune detection was performed using an ECL kit according to the manufacturer's directions (Amersham Pharmacia Biotech).

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[0037] Generally, the present invention provides a construct encoding a soluble β2m/HFE monochain or a functional derivative or a salt thereof. Preferably, the protein encompasses the human β2m or an analog or active fragment thereof, an artificial linker that preferably encodes 15 amino acids, and the α1-α3 domains of the human HFE protein or an analog or active fragment thereof. The protein is capable of binding TfR, being internalized by cells, and regulating iron metabolism. This protein, when conjugated to a desired agent, is capable of targeting specific cells and delivering drugs into cells.

[0038] The term "monochain" as used herein is meant to include, but is not limited to, recombinant polypeptides that are linked via a compatible linker to provide a single recombinant protein.

[0039] The term "soluble" as used here is meant to include, but is not limited to, recombinant proteins or polypeptides that are not directly anchored in cellular membranes.

[0040] The term "therapeutic" as used herein is meant to include, but is not limited to, toxic agents, chemotherapeutic drugs, peptides, polypeptides, proteins, chemical compounds, other desired therapeutics or DNA, or any molecular marker that can be attached to a soluble monochain β2m/HFE protein.

[0041] As indicated above, the soluble β2m/HFE monochain is composed of three parts. The first part is human β2-microglobulin (β2m) whose sequence is known and available at GenBank Accession Nos. NP_004039 and XP_032402. The full sequence of β2m appears as residues 1-119 of SEQ ID NO:2. It should be understood that analogs and active fragments of this sequence can be substituted for the β2m portion of the monochain in accordance with the present invention. The analog or active fragment must retain the properties of β2m that permit the bioactivity of the monochain of the present invention as disclosed herein, i.e., the β2m portion must confer stability and property conformation required for trafficking and function of the formed monochain iron regulation.

[0042] The HFE portion of the monochain is, preferably, the α1-α3 domains of the human HFE protein, i.e., amino acids 134-409 of SEQ ID NO:2. Again, analogs and functional derivatives of this sequence may be substituted, as long as the resulting polypeptide interacts with the β2m portion of the monochain so as to permit the formed monochain to bind TfR and to regulate of iron metabolism in target cells.

[0043] The linker portion must be sufficiently flexible to allow the active complex to be maintained. The preferred flexible linker is made of GGGGS (SEQ ID NO:15) repeats. The optimum number of repeats has been determined to be three, although any number of repeats from 1 to 5 may be employed in

the monochain. Other flexible linkers, as are well known in the art, may be substituted for the linker described herein as the preferred embodiment. Such a flexible linker, is preferably, about 15 amino acids in length, although linkers of from 5 to 25 amino acids can be devised as long as they do not form a particularly immunogenic epitope between the β2m and the HFE portions of the monochain and as long as they do not inhibit the activity of the monochain as disclosed herein. Preferably, such a linker will have a neutral charge so as not to affect the conformation of the monochain.

[0044] The flexible linker may have an intracellular protease cleavage site. Alternatively, an intracellular protease cleavage site may be located adjacent to the linker. A variety of proteases are released by necrosing and apoptosing cells. Included in these are caspases (Interleukin 1 beta-converting enzyme-like proteases), metallo proteinases, lysosomal proteases (e.g., cathepsin B) and elastase. Elastase is released by granulocytes during disease states (e.g., sepsis) and has a broad specificity regarding amino acid cleavage sequence, akin to that of trypsin (Ertel et al, 1994; Szilagyi et al, 1995).

[0045] Intracellular proteases can act on the soluble β2m/HFE monochain containing an intracellular protease recognition sequence in the linker portion to allow the β2m and the HFE portions to be separated once delivered into the cell in order to enhance, to stabilize or to promote other functions of the monochain associated with iron metabolism.

[0046] An analog of β2m or HFE as described herein has an amino acid sequence essentially corresponding to that of the native human β2m or the specified domains of native HFE. The term "essentially corresponding to" is intended to comprehend analogs with minor changes to the native sequence of the specified polypeptide, which changes do not affect the basic characteristics thereof, particularly insofar as its ability to

be used in the monochain of the present invention, which monochain binds to TfR and regulates iron absorption. The type of changes which are generally considered to fall within the "essentially corresponding to" language are those which would result from conventional mutagenesis techniques of the DNA encoding the β2M or HFE, resulting in a few minor modifications, and screening for the desired activity in the manner discussed above.

[0047] Preferably, the analog is a variant of the native sequence, which variant has an amino acid sequence having at least 70% identity to the native amino acid sequences and retains the biological activity thereof. More preferably, such a sequence has at least 85% identity, at least 90% identity, or most preferably at least 95% identity to the native sequences.

[0048] The term "sequence identity" as used herein means that the sequences are compared as follows. The sequences are aligned using Version 9 of the Genetic Computing Group's GAP (global alignment program), using the default (BLOSUM62) matrix (values -4 to +11) with a gap open penalty of -12 (for the first null of a gap) and a gap extension penalty of -4 (per each additional consecutive null in the gap). After alignment, percentage identity is calculated by expressing the number of matches as a percentage of the number of amino acids in the claimed sequence.

[0049] Analogs in accordance with the present invention may also be determined in accordance with the following procedure. Polypeptides encoded by any nucleic acid, such as DNA or RNA, which hybridizes to the complement of the native DNA or RNA under highly stringent or moderately stringent conditions, as long as that polypeptide maintains the biological activity of the native sequence are also considered to be within the scope of the present invention. The native DNA sequence for β2m is that part of SEQ ID NO:1 that encodes the β2m portion of the monochain, i.e., nucleotides 1-357 of SEQ ID NO:1. The native

DNA for the portions of HFE in the monochain is that part of SEQ ID NO:1 which encodes the HFE portion of the monochain, i.e., nucleotides 403-1227 of SEQ ID NO:1.

[0050] Specificity in hybridization, however, is a function of the difference in stability between the desired hybrid and "background" hybrids. Hybrid stability is a function of duplex length, base composition, ionic strength, mismatching, and destabilizing agents (if any).

[0051] The Tm of a perfect hybrid may be estimated for DNA:DNA hybrids using the equation of Meinkoth et al (1984), as

$$Tm = 81.5°C + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$$

and for DNA:RNA hybrids, as

$$Tm = 79.8°C + 18.5 (\log M) + 0.58 (\%GC) - 11.8 (\%GC)^2 - 0.56(\% \text{ form}) - 820/L$$

where

M, molarity of monovalent cations, 0.01-0.4 M NaCl,

%GC, percentage of G and C nucleotides in DNA, 30%-75%,

% form, percentage formamide in hybridization solution, and

L, length hybrid in base pairs.

[0052] Tm is reduced by 0.5-1.5°C (an average of 1°C can be used for ease of calculation) for each 1% mismatching.

[0053] The Tm may also be determined experimentally. As increasing length of the hybrid (L) in the above equations increases the Tm and enhances stability, the full-length rat gene sequence can be used as the probe.

[0054] Filter hybridization is typically carried out at 68°C, and at high ionic strength (e.g., 5 - 6 X SSC), which is non-stringent, and followed by one or more washes of increasing stringency, the last one being of the ultimately desired high stringency. The equations for Tm can be used to estimate the

appropriate Ti for the final wash, or the Tm of the perfect duplex can be determined experimentally and Ti then adjusted accordingly.

[0055] Hybridization conditions should be chosen so as to permit allelic variations, but avoid hybridizing to other genes. In general, stringent conditions are considered to be a Ti of 5°C below the Tm of a perfect duplex, and a 1% divergence corresponds to a 0.5-1.5°C reduction in Tm.

[0056] As used herein, highly stringent conditions are those which are tolerant of up to about 5% sequence divergence. Without limitation, examples of highly stringent (5-10°C below the calculated Tm of the hybrid) conditions use a wash solution of 0.1 X SSC (standard saline citrate) and 0.5% SDS at the appropriate Ti below the calculated Tm of the hybrid. The ultimate stringency of the conditions is primarily due to the washing conditions, particularly if the hybridization conditions used are those which allow less stable hybrids to form along with stable hybrids. The wash conditions at higher stringency then remove the less stable hybrids. A common hybridization condition that can be used with the highly stringent to moderately stringent wash conditions described above is hybridization in a solution of 6 X SSC (or 6 X SSPE), 5 X Denhardt's reagent, 0.5% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA at an appropriate incubation temperature Ti.

[0057] The term "active fractions" of the native polypeptide or analog thereof is intended to cover any fragment thereof that retains the biological activity, as explained hereinabove for each of β2m and HFE in the definition of "analog". For example, fragments can be readily generated from the full-length sequence where successive residues can be removed from either or both the N-terminus or C-terminus thereof or from peptides obtained therefrom by enzymatic or chemical cleavage of the polypeptide. Thus, multiple substitutions are not

involved in screening for active fractions. If the removal of one or two amino acids from one end or the other does not affect the biological activity, such truncated polypeptides are considered to be within the scope of the present invention. Further truncations can then be carried out until it is found where the removal of another residue destroys the biological activity.

[0058] "Functional derivatives" as used herein covers chemical derivatives which may be prepared from the functional groups which occur as side chains on the residues or the N- or C-terminal groups, by means known in the art, and are included in the invention as long as they remain pharmaceutically acceptable, i.e., they do not destroy the biological activity of the corresponding polypeptide, or the associated monochain, as described herein and do not confer toxic properties on compositions containing it. Derivatives may have chemical moieties, such as carbohydrate or phosphate residues, provided such a fraction has the same biological activity and remains pharmaceutically acceptable.

[0059] Suitable derivatives may include aliphatic esters of the carboxyl of the carboxyl groups, amides of the carboxyl groups by reaction with ammonia or with primary or secondary amines, N-acyl derivatives or free amino groups of the amino acid residues formed with acyl moieties (e.g., alkanoyl or carbocyclic aroyl groups) or O-acyl derivatives of free hydroxyl group (e.g., that of seryl or threonyl residues) formed with acyl moieties. Such derivatives may also include for example, polyethylene glycol side-chains that may mask antigenic sites and extend the residence of the complex or the portions thereof in body fluids.

[0060] Non-limiting examples of such derivatives are described below.

[0061] Cysteinyl residues most commonly are reacted with alpha-haloacetates (and corresponding amines), such as

chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, alpha-bromo- beta-(5-imidazoyl)propionic acid, chloroacetyl phosphate, - alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl-2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4- nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

[0062] Histidyl residues are derivatized by reaction with diethylprocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Parabromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

[0063] Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing alpha-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2, 4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

[0064] Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclodexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

[0065] The specific modification of tyrosyl residues *per se* has been studied extensively, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most

commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

[0066] Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R'-N-C-N-R') such as 1-cyclohexyl-3-[2-morpholinyl-(4-ethyl)]carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethlypentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

[0067] Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

[0068] The term "derivatives" is intended to include only those derivatives that do not change one amino acid to another of the twenty commonly-occurring natural amino acids.

[0069] The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the monochain of the invention or analogs thereof. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, salts with mineral acids, such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids, such as, for example, acetic acid or oxalic acid. Of course, any such salts must have substantially similar biological activity to the monochain of the invention.

[0070] In addition, the soluble β2m/HFE monochain can be tagged for ease of detection and purification. For instance, a

His-tag, which is a stretch of 6 histidines at the C-terminus, is recognized by commercial antibodies and thus serves as a tag for immune detection. It provides some other advantages of affinity purification and concentration of the recombinant protein from supernatants, cell lysates and bacterial lysates. The affinity purification is based on the specific interaction of the target protein (through the histidine tag) with an immobilized ligand (Nickel beads, metal-based chromatography). The addition of the his-tag to recombinant proteins provides a number of affinity purification strategies that may be applied to sort out a specific recombinant protein from a mixture, to capture the desired molecule for interaction studies or to remove a component from the purified recombinant protein preparation, such as protease removal in cleavage reactions. For affinity purification strategies, other tags can be used such as the GST-tag, T7-tag. S-tag, and CBD-tag, which can all be purified by specific resins, such as those mainly provided by Novagene.

[0071] The myc-tag, which has the myc-tag epitope Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu (residues 418 to 427 of SEQ ID NO:2), can be used to enable the detection of the recombinant soluble monochain in cell lysates and/or supernatants in order to follow up expression and secretion. This tag is recognized by a well known commercial antibody that allows the detection of the recombinant protein, a detection which is not dependent on conformation as opposed to antibody 2F5, which is conformationally dependent and serves only for limited specific assays.

[0072] A protease cleavage site, such as that for Factor Xa, can be inserted at the N- or C-terminus of the soluble monochain in order to remove the his-tag or other tags after the purification steps and before the injection of the soluble β2m/HFE monochain. This step might be required to eliminate a

possible immune response against the tag itself once injected into the circulation.

[0073] A recombinant construct of the soluble β2m/HFE monochain according to the present invention can be prepared by recombinantly fusing the native DNA sequence encoding the β2m, i.e., nucleotides 1-357 of SEQ ID NO:1, or an active fraction thereof via a DNA sequence encoding the linker portion to a native DNA sequence encoding for the HFE portion, i.e., nucleotides 403-1227 of SEQ ID NO:1, or an active fraction thereof using conventional well-known recombinant DNA techniques. Alternatively, either or both of β2m and HFE in the mon0chan may be encoded by a DNA which hybridizes to the native DNA sequences or can be obtained by hybridization to the native DNA sequences under highly stringent or moderately stringent conditions. The recombinant construct can be placed into an expression vector under the control of an appropriate promoter for expression of the soluble β2m/HFE monochain in a host cell. The host cell, which is preferably eukaryotic but may be prokaryotic, when transformed with the expression vector can be used for preparing the soluble β2m/HFE monochain. This method of preparing the soluble β2m/HFE monochain according to the present invention involves culturing the transformed host cell to express and produce the soluble β2m/HFE monochain and then recovering the produced monochain product.

[0074] The recombinant construct encoding the protein described above, may serve as a platform for efficient drug delivery into cells and for modulation of iron absorption/ metabolism. Since the TfR is over-expressed in tumors, metastases, activated lymphocytes operative in autoimmune diseases, and inflammatory disorders, this platform has several advantages over current therapies. Particularly, it will be able to modulate iron metabolism and, consequently, arrest cell proliferation and activation. In addition, since the monochain is soluble, but as stable as the natural HFE molecule, it could

be administered for treatment of iron overload disorders and the like.

[0075] To use the monochain as a platform for drug delivery, a therapeutic may be bound to the monochain. The bond may be, for example, a covalent bond or a peptide bond. In each instance, however, a simple assay may be conducted to determine that the assembled monochain-therapeutic retains the ability of the monochain to bind TfR. Preferably, the therapeutic is a protein or polypeptide which is fused to the monochain via a peptide bond. In a preferred embodiment, it is fused to the C-terminus of the monochain. The experiments herein establish that an additional protein, which is green fluorescent protein (GFP) in the examples, can be fused to the C-terminus of the monochain without affecting the ability of the monochain to bind TfR. The therapeutic may also be fused to the N-terminus as long as it is experimentally established that it does not so affect the conformation of the monochain that it no longer binds TfR.

[0076] When the monochain-therapeutic is a fusion protein, it is preferably made by designing an appropriate DNA construct which encodes the fusion protein. The monochain-therapeutic in which the therapeutic is a protein or polypeptide fused to the soluble β2m/HFE monochain by a peptide bond can either be constructed by fusing the DNA encoding the therapeutic protein or polypeptide to the recombinant DNA construct of the soluble β2m/HFE monochain or be constructed by fusing the therapeutic protein or polypeptide after the soluble β2m/HFE monochain has been prepared using conventional peptide chemistry techniques. When the recombinant DNA construct of the soluble β2m/HFE monochain is fused to the DNA encoding the therapeutic protein or polypeptide to the monochain to generate a further construct, this further construct can be placed into an expression vector under the control of an appropriate promoter for expression of the monochain-therapeutic in a host cell.

The host cell, which is preferably eukaryotic but may be prokaryotic, when transformed with the expression vector can be used for preparing the monochain-therapeutic. This method of preparing the monochain-therapeutic according to the present invention involves culturing the transformed host cell to express and produce the monochain-therapeutic and then recovering the produced monochain-therapeutic product.

[0077] A target for proteolytic cleavage by intracellular proteases, preferably in the endosomes and in the cytosol, can be introduced, i.e., recombinantly in the above monochain-therapeutic, between the therapeutic drug and the soluble β2m/HFE monochain in order for the transported therapeutic drug to be cleaved immediately after it penetrates the cell and to exert its function. Most transported substances require interaction with their specific target at specific locations inside the cell and, hence, need to be released from the monochain carrier.

[0078] The genetically engineered monochain of the present invention can be utilized as a platform for advanced delivery of drugs and other chemicals into cells, and for the regulation of iron metabolism in target cells. This protein encompasses two partners:

1. The β2m molecule that confers stability and proper conformation required for trafficking and function in iron regulation.
2. A polypeptide derived from the HFE protein that is essential for TfR binding and for the regulation of iron metabolism in target cells.

[0079] The necessity for such a system is apparent, especially when violent cancer cells become drug resistant and escape to the central nervous system, which is inaccessible to most chemotherapeutics. Drug delivery to the brain and down-regulation of iron consumption of metastasizing cells is expected to be a solution to these specific problems.

Moreover, in cases where autoimmune cells are activated or in graft implantations, biotechnological tools that down-modulate such cells are required. The present data show that most tumor cell lines, as well as activated lymphocytes express constitutively high levels of TfR, the receptor that is targeted by β2m/HFE monochain.

[0080] Three forms of the recombinant protein were engineered for different purposes. Two forms harbor a His-tag at the N-terminus (β2m/HFE/Mh) and at the C-terminus (β2m/HFE/Mhm) of the molecule. The third form harbors a Green Fluorescent Protein (GFP) (Zhang et al, 1996) protein that is fused to the C-terminus of the monochain (β2m/HFE/Mg). The different forms of the monochain are expressed in HEK293 cells and are secreted to the surrounding media. Both the β2m/HFE/Mh and β2m/HFE/Mhm molecules bind to TfR with similar affinities; however, only the β2m/HFE/Mhm protein is properly folded, as verified by its recognition with conformation-dependent antibody, 2F5. The fact that the binding of β2m/HFE to TfR is independent of proper conformation will allow the conjugation of peptides or polypeptides to the N-terminus of the monochain despite their potential to affect β2m/HFE conformation. Such molecules could deliver drugs but might not have a function in iron metabolism. The β2m/HFE/Mhm and β2m/HFE/Mg molecules are soluble and stable in a serum-rich medium. Therefore, apparently, they are resistant to serum proteases, an advantageous property for a drug delivery vehicle that should be administered into the circulation. When synthesized in cells or added to human cell lines in the culture media, both β2m/HFE/Mhm and β2m/HFE/Mg molecules penetrate target cells rapidly, within 1 hour. When β2m/HFE monochain is chemically linked to another molecule (FITC conjugated molecule) or synthesized as an engineered protein (GFP monochain), it is able to deliver chemicals or proteins into cells. β2m/HFE monochain administered in the culture media, penetrates

efficiently into human cells in a transferrin-dependent manner and co-localizes, at least partially, with transferrin containing vesicles. Therefore, the monochain and its derivatives specifically target TfR on the surface of human tumors, and could reach the endosomes, the desired intracellular target organelle. The present data also indicate that the β2m/HFE/Mg reaches other intracellular organelles. Interestingly, the results imply that the human TfR requires co-binding of its natural ligand, the human transferrin, in order to promote efficient binding of the monochain to TfR or efficient internalization of monochain-TfR complexes. Since transferrin is present in large excess in the circulation, it is not a limiting factor for the binding of β2m/HFE monochain to the TfR.

[0081] The β2m/HFE monochain is able to target and deliver drugs into human TfR-expressing cells. Since transferrin-based drug delivery was shown to overcome drug resistance of tumor cells and also to penetrate the brain, it is expected that β2m/HFE monochain-based therapy will operate in a similar manner.

[0082] Peptide and protein therapeutics are generally excluded from transport from blood to brain, owing to the negligible permeability of these drugs to the brain capillary endothelial wall, which makes up the blood-brain barrier *in vivo*. However, peptides or protein therapeutics may be delivered to the brain when a non-transportable therapeutics is coupled to a BBB drug transport vector. The principle of the drug delivery into the brain tissue is when the transport vector binds to the luminal plasma membrane of endothelial cells via the interaction of the transport vector with a cell surface receptor. The binding step then initiates transcytosis of the whole complex across the endothelial cell and results in the release of the transported vector into the brain extracellular fluid. When a cleavable linker between the

transport vector and the drug, such as disulfide bond is employed, tissue enzymes may cleave the drug from the vector.

[0083] Receptor-mediated transcytosis across the BBB has to date only been shown for insulin and transferrin where the latter is necessary for the cellular delivery of iron into the brain. Binding of transferrin to the TfR at the cell surface membrane, leads to internalization of the transferrin in vesicles, that are further headed for exocytosis at the luminal plasma membrane, via vesicular trafficking through intracellular compartments. Due to its high endogenous plasma concentration of 25 uM, transferrin itself may be limited as a drug transporting vector, since the transferrin receptors are almost saturated under physiologic conditions. As an alternative approach, anti-TfR antibody was demonstrated to be an efficient transport vector, although it presents problems of immune response. A different ligand of the TfR, the soluble β2m/HFE monochain, that is not affected by the transferrin-saturation condition of the receptor and undergoes internalization as the transferrin does, would serve as an optimal BBB vector.

[0084] Since the β2m/HFE monochain was engineered from two polypeptides operating in concert to down-modulate iron uptake, opposite to the effect of transferrin, this molecule is advantageous over the transferrin molecule. The main outcome of treatment with β2m/HFE monochain is the decreased level of TfR in treated cells. This result is compatible with another result: following 2 hours of treatment of cells with β2m/HFE/Mg there was hardly any detectable level of β2m/HFE monochain residing in cells. Since at 2 hours of incubation the monochain was hardly detected in cells, although it was present in large excess in the conditioned medium, it is highly feasible that the complex monochain/TfR was directed towards degradation. This process would result in decreased level of functional TfR on the cell surface. It appears that following

the binding to its cognate receptor, the β2m/HFE monochain leads the TfR to degradation, thereby preventing further TfR-dependent iron uptake.

[0085] The conclusion that β2m/HFE monochain affects iron metabolism is clearly supported by the results of the attempt to over-express HFE and β2m/HFE monochain in tumor cell lines. It was observed that over-expression of HFE as well as β2m/HFE in HeLa and PC3 human tumor cell lines confers a yet unidentified stress resulting in the rapid degradation or blocking of synthesis of HFE and β2m/HFE. Since TfR is the main iron uptake system in dividing cells, it is expected that the expression of HFE-derived molecules could inhibit iron uptake and, consequently, cause growth arrest and death. These data strongly corroborate the conception that the monochain can be used as a biotechnological tool for causing growth arrest of highly proliferating cells.

[0086] While the examples of the present invention relate mostly to cancer therapy and treatment of autoimmunity, the monochain could be used in any condition that would benefit from such a treatment.

EXAMPLE 1: General Techniques

Cell Culture

[0087] Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, GibcoBRL, Life Technologies Ltd, Palsley, Scotland), supplemented with 2 mM glutamine (Biological Industries, Beit Haemek, Israel), 10% fetal calf serum (FCS, HyClone Laboratories, Inc, Logan, Ut, USA), penicillin, streptomycin, gentamycin and amphotericin B (Biological Industries, Beit Haemek, Israel). For certain experiments, as well as for purification of the monochain molecule from culture medium, a low protein synthetic medium DCCM-1 (Biological Industries, Beit Haemek, Israel) was used.

Cell Lines

[0088] The following cell lines were used: human embryonal kidney HEK293 (ATCC), mouse embryonal fibroblasts transformed with adenovirus 12, VAD12.79, mouse melanoma, B16, HeLa (ATCC), human neuroblastoma cell lines, SK-NSH, Nub7, NHB, WN, human hepatoma cell lines Hep-G2, SK-HEP1, HEP3bp5, HuH7, human cervical squamous carcinoma, SiHa, human cervical epidermal carcinoma, Caski, human melanoma cell lines GTBS, WUBI, FO1, human ovarian carcinoma cell lines HEY, HEYC2, OVCAR, OC109, human breast carcinoma cell lines T47D, MDA 231, human squamous carcinoma cell lines 22A, 14C-16, human Burkitt's lymphoma cell lines, Daudi, DG-75, human pre B, Nalm6, human T leukemia, Jurkat, human chronic myelogenous Leukemia cell lines K562, Dami, MegO1, human promyelocytic leukemia HL-60.

Antibodies

[0089] The following monoclonal antibodies (mAbs) were used; R17 217.1.3 (Lesley et al 1984) (anti-mouse TfR, ATCC TIB-219), S19 (Tada et al, 1980) (anti-mouse β2m, a kind gift of Dr. T. Hansen, Washington University, St Louis), BBM1 (anti-hβ2m, ATCC HB-28), anti-HFE (CT) (rabbit anti-hHFE antisera prepared by immunization of rabbits with peptide (C)RKRQGSRGAMGHYVLAERE (SEQ ID NO:3) that corresponds to the sequence of the cytoplasmic tail of hHFE), B2.62.2, anti hβ2m, kindly provided by F. Lemonnier, Unité d'Immunité Cellulaire Antivirale, Insititut Pasteur, Paris, France, V1-10 (anti-human TfR, a kind gift of Dr. Z. Eshhar, The Weizmann Institute of Science, Rehovot, Israel), H68.4, anti-TfR (mouse and human) Zymed Laboratories, San Francisco, CA), anti MAP kinase polyclonal antibodies (442704-S) (Sigma), anti myc tag (9E10 hybridoma) (Fan et al, 1998).

Northern Blot Analysis and RT-PCR

[0090] RNA extraction and Northern blot analysis were performed as previously described (Rotem-Yehudar et al, 1994, Fromm et al, 1998). Reverse transcription of RNA was carried

out for 1 hour at 42°C, in 20 µl PCR buffer containing 1 µg of total cellular RNA, 0.5 µg Oligo d(T)18 primer (Promega, Madison, WI, USA), nucleotide mixture (1 mM), 24 units ribonuclease inhibitor (MBI FermentasVilnius, Lithuania), and 15 units AMV reverse transcriptase (Promega). PCR was carried out in the appropriate buffer with 1 µl of the cDNA, 25-50 pmole of the relevant primers, 40 µM nucleotide mixture, and 1.54 units Taq DNA polymerase (Biological Industries). After the appropriate number of PCR cycles (MiniCycler, MJ Research Inc. Watertown, MA, USA), 10 µl or less of the reaction mix were fractionated on a 1% agarose gel. Specific conditions are described in the section "Specific Techniques".

Primers

Actin- Forward 5′ GTTTGAGACCTTCAACACCCC 3′ (SEQ ID NO:4)

Reverse 5′ GTGGCCATCTCTTGCTCGAAGTC 3′ (SEQ ID NO:5)

HFE- Forward 5′ GGGGCGCTTGCTGCGTTCACAC 3′ (SEQ ID NO:6)

Reverse 5′ GGTCCAAAACACCTCTCCCCAGCTCC 3′ (SEQ ID NO:7)

[0091] Other specific primers are described in the "Specific Techniques" section.

FACS® Analysis

[0092] Cells were harvested by mild trypsinization, followed by washes in media supplemented with 5% FCS and 0.01% sodium azide. About 10^6 cells were incubated at 4°C with the appropriate concentration of the first antibody for 45 minutes, washed and then incubated in the dark for another 30 minutes with the second antibody. The cells were then washed with PBS and fluorescence intensity was analyzed with a Becton-Dickinson Cell Sorter (Becton-Dickinson & Co., Mountain View, CA, USA).

Metabolic Labeling, Immunoprecipitation and Western Blot Analysis

[0093] Cells were grown to 80% confluence and starved for 60 minutes in methionine-free medium. They were then labeled in methionine-free medium containing 150 µCi/ml ^{35}[S]-methionine

(Amersham Pharmacia Biotech, Little Chalfont, UK) for 30 minutes, washed with PBS and chased for the indicated intervals. The cells were lysed with buffer containing 0.5% NP-40, 50 mM Tris (pH 7.5), and 150 mM NaCl, immunoprecipitated with the indicated antibodies and Protein A (Boehringer Mannheim GmbH). The immunoprecipitates were washed with buffer containing 0.1% NP-40, 50 mM Tris (pH 7.5), and 150 mM NaCl. For endo H-treatment, immunoprecipitates were eluted by adding 20 µl buffer containing 100 mM Tris (pH 8), 1% SDS and boiling for 5 minutes. The samples were centrifuged and the supernatant was added to 2 µl of 50 mM citrate buffer (pH 5.5) containing 1000 units of endo H (New England Biolabs, Beverly, MA, USA). The samples were incubated at 37°C for 18 hours, followed by the addition of sample buffer. All the buffers contained the following protease inhibitors: 1 mM PMSF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin (Sigma Chemical Co.). The protein concentration was determined with a Bicinchronic Acid Protein Assay Kit (Sigma Chemical Co.) and equivalent amounts of protein were loaded on the gels. Following fractionation of the immunoprecipitates on 12-15% SDS-PAGE, X-OMAT AR X-ray films (Eastman Kodak Company, Rochester, NY, USA) were exposed to the dried gels.

[0094] For Western blot analysis, the immunoprecipitates or cell extracts were transferred to nitrocellulose (Amersham Pharmacia Biotech). The membrane was hybridized with the specific antibody, followed by the appropriate horseradish peroxidase-conjugated secondary antibodies and chemiluminescence was measured (Amersham Pharmacia Biotech) according to the manufacturer's directions.

Quantitation of Radioactive Signals

[0095] Dried radioactive gels or blots were exposed to a phosphoimager screen and analyzed with a phosphoimager (Fuji BAS1000, Tokyo, Japan).

Immunocytochemistry

[0096] Sub-confluent cells grown on coverslips (11 mm:Menzel glass, Merk, Germany) were incubated with either 20 µg/ml of β2m/HFE monochain or 5 µg/ml of tetramethylrhodamine-conjugated transferrin (Molecular Probes). The cells were washed twice with PBS (pH 7.4) and fixed in methanol for 5 minutes at 4°C and in methanol/acetone (1:1) for 5 minutes at 4°C. The cells were washed 3 times for 5 minutes in PBS. The coverslips were incubated for 60 minutes with anti-human β2m antibodies (B2.62.2) followed by 3 washes of 5 minutes with PBS. The coverslips were incubated for 60 minutes with Cy2-conjugated goat anti mouse antibodies or Cy3-conjugated goat anti mouse antibodies (Jackson laboratories), washed 4 times for 5 minutes with PBS, mounted with Gel-Mount (Biomeda Corp.) and then analyzed by immunofluorescence microscopy and Confocal microscopy.

EXAMPLE 2: Specific Techniques and Generation of Tools for Physical, Biochemical and Functional Analysis of the β2m/HFE Monochain

Production of Anti-hHFE Monoclonal Antibodies

[0097] The hHFE-transfected clone of VAD12.79 that expresses the human HFE (WH6) (generated in Dr. Ehrlich laboratory) was used for syngeneic immunization of C57Bl/6 mice. The mice received one subcutaneous injection of 10^6 cells in CFA and then five additional injections (one injection every 4 weeks) of 10^6 cells each. One day prior to removal of the spleen, the mice were immunized intravenously with 10^5 irradiated cells. Hybridization of the spleen cells with NS0 myeloma cells, subsequent culture, and cloning were performed as described (Kohler et al, 1975). Monoclonal antibodies (mAbs) were selected according to their specific binding to WH6 cells as analyzed by FACS. The mAb 2F5, directed against a

conformation-dependent epitope on HFE/β2m heterodimer was utilized for further immunoprecipitation and FACS analyses.

Construction of Plasmids Encoding Soluble β2m/HFE Monochain

[0098] A single-chain HFE gene expression vector in which a full-length β2m gene linked via its 3′ end (the region encoding the carboxy-terminus of the molecule) to the 5′ end (the region encoding the amino-terminus of the α1-α3 domains) of the human HFE gene through an oligonucleotide spacer (encoding the 15-amino acid peptide $(GGGGS)_3$ (SEQ ID NO:8)), was constructed. The construct was obtained by splicing overlap extension PCR. The chimeric PCR product was introduced into BamHI-XhoI sites of the mammalian expression vector pcDNA3.1/his C (Invitrogene) and into BamHI-XhoI sites of the mammalian expression vector pcDNAHis/Myc A (Invitrogene). The β2m was amplified from a plasmid harboring human β2m gene, with a 5′ primer (P1) that incorporates a BglII restriction site followed by the 5′ end of the β2m leader sequence, and a 3′ primer (P2) that incorporates the 3′ terminus of the β2m coding region and 39 bases encoding the 5′ region of the peptide spacer.

P1: 5′ ggcacgaagatctgagatgtctcgctccgtgg 3′ (SEQ ID NO:9) (The BglII site is underlined.)

P2: 5′ gccgccaccggatccacctccgcccgagccgccacctccatgtctcgatcccacttaac 3′ (SEQ ID NO:10) (The BamHI site and β2m terminus are underlined respectively.)

[0099] The human HFE cDNA was isolated from human liver cDNA library. Following amplification with the relevant primers, the 5′ primer, P3, and the 3′ primer, P4. P3 encodes 18-bp overlap with the 3′ end of P2, 6 bases encoding the 3′ region of the spacer, and the 5′ region of the HFE α1 domain. P4 encodes the 3′ end of the HFE α3 domain and an XhoI restriction site.

P3: 5′ ggctcgggcggaggtggatccggtggcggcggttcccgcttgctgcg ttcacac 3′ (SEQ ID NO:11;) (The BamHI restriction site and the α1 domain are underlined, respectively.)

P4: 5′ gccagacggctcgagctcccagatcacaatgagggg 3′ (SEQ ID NO:12) (The XhoI site is underlined)

[0100] The P3/P4 HFE amplification product was mixed in a splicing overlap extension PCR with the P1/P2 β2m gene product following by amplification with the 5′ primer P1 and the 3′ primer P4. This amplification resulted in a single construct incorporating the leader - β2m - spacer - HFE (α1-α3) coding sequences flanked by 5′ BamHI and 3′ XhoI restriction sites. P1/P2, P3/P4, PCR were carried out as follows: 30 cycles of 95°C for 90 seconds, 60°C for 1 minute, and 72°C for 2 minutes. The final overlapping PCR was performed by a single cycle of 95°C for 90 seconds, 50°C for 1 minute, and 71°C for 150 seconds without primers to facilitate annealing of the β2m and HFE fragments, following by 45 cycles of 95°C for 90 seconds, 60°C for 1 minute, and 72 °C for 160 seconds in the presence of primers P1 and P4. The final PCR product was enzymatically cleaved by BglII-XhoI sites and was ligated in frame into pcDNA3.1/His and pcDNAHis/Myc (Invitrogene) using the BamHI/BglII-XhoI cloning sites. The correct sequence of the full-length insert was verified by sequencing using relevant primers. β2m/HFE monochain in pcDNA3.1/His is designated β2m/HFE/Mh. β2m/HFE monochain in pcDNAHis/Myc is designated β2m/HFE/Mhm.

Construction of a Three Partner-Monochain Encoding a GFP/Monochain Chimera

[0101] A monochain of human soluble β2m-HFE-EGFP(His/Myc) was constructed by cloning in frame cDNA encoding EGFP (Zhang et al, 1996) into the β2m/HFE/Mhm expression vector. The EGFP was cloned downstream of the β2m/HFE monochain sequence but upstream of the His/Myc epitope sequences. EGFP cDNA was

amplified by PCR from pEGFP.C2 vector (Clontech Laboratories Palo Alto, CA, USA) using the primers:

EGFP Forward – 5′ CCATCTCGAGGTCGCCACC**ATGGTGAG** 3′ (SEQ ID NO:13)

EGFP Reverse – 5′ GCTCTAGAGGA**CTTGTACAGCTCGTCC** 3′(SEQ ID NO:14)

[0102] The underlined sequences indicate restriction digest sites while bold sequences demonstrate EGFP coding sequences.

[0103] The EGFP Forward primer incorporated XhoI restriction site and leader sequence from EGFP, while EGFP Reverse primer included Xba1 restriction site and sequence encoding for the carboxy terminus of EGFP excluding STOP codon. EGFP PCR product was amplified in a standard PCR reaction buffer with 2.5 U of Taq polymerase (Takara Shuzo, Shinga, Japan) at 30 cycles of the following PCR condition: denaturation at 94°C for 30 seconds; annealing at 55°C for 30 seconds and; extension at 72°C for 30 seconds. The PCR product was digested with XhoI and Xba1 and cloned in frame into the corresponding sites in the β2m/sHFE/Mhm vector. The resulting β2m/HFE/GFP vector was analyzed by a diagnostic restriction digest and correct sequence confirmed by automated sequencing. β2m/HFE monochain in pGFP is designated β2m/HFE/Mg.

Stable Transfection

[0104] HEK293, HeLa and VAD12.79, were transfected by calcium phosphate DNA co-precipitation as described (Fromm et al, 1998). The plasmid DNA contained either 10 µg of the hHFEcDNA (human HFE), mutant hHFEcDNA (C282Y), β2m/HFE/Mhm, β2m/HFE/Mh and β2m/HFE/Mg or human TfR cDNA. Individual clones were selected and expanded as described previously (Fromm et al, 1998). The stably transfected cells are designated as follows: 293/mutHFE, 293/HFE, 293/β2m/HFE/Mh, 293/β2m/HFE/Mhm, 293/β2m/HFE/Mg, HeLa/β2m/HFE/Mhm, HeLa/mutHFE, HeLa/HFE, and VAD12.79/hTfR.

Purification of Secreted β2m/HFE Monochain Protein from Conditioned Media

[0105] 300-500 ml of DCCM-1 supernatants collected from 293/β2m/HFE/Mhm-secreting cells, were incubated with 10 ml of His-bind beads (Novagene) at 4°C for 2 hours, followed by washing with 300 ml of phosphate-buffered saline (PBS) at pH 7.4. Elution was carried out using 5 ml of 200 mM of Immidazole in PBS at pH 7. Eluates were tested for protein concentration by bicinchoninic acid (BCA) assay (Pierce), dialyzed against PBS and fractionated on SDS-PAGE.

Chemical Linkage of FITC to Purified β2m/HFE Monochain

[0106] Isothiocyanate labeling procedure was carried out as described in Antibodies: A Laboratory Manual (Harlow and Lane, 1998 (Chapter 9, pp. 354-355). 200 µg of purified protein was adjusted to pH 9 in 0.1 M sodium carbonate. Fluorescein isothiocyanate (FITC) (Sigma) was freshly dissolved in 1 mg/ml dimethyl sulfoxide and 10 µl of FITC solution was added to the protein. The reaction was carried out in the dark for 8 hours at 4°C. NH_4Cl was added to a final concentration of 50 mM and unbound FITC was separated from the conjugate by dialysis against PBS for 48 hours at 4°C.

RESULTS

Expression of HFE and TfR in Human Tumor Cell Lines and in Human Activated Lymphocytes

[0107] 30 human tumor cell lines of different origin have been screened for the expression of HFE and TfR genes by Northern blot analysis (Figs. 1a-1c). The data demonstrate that, except for neuroblastomas, all the tumors express TfR. Tumors of lymphoid origin, melanomas, cervical carcinomas, breast cancer and some hepatomas express very high TfR levels. The expression of HFE is variable and does not directly correlate with TfR expression. The TfR signal was evident following 4h exposure of the labeled blot to X-ray film, while

the HFE signal was detected following 2 weeks exposure of the same blot to X-ray film. Thus, it is apparent that HFE expression in human tumors is relatively low while the TfR expression is remarkably high. These results imply that the TfR is the main iron uptake system in tumors, and HFE is its adversary negative regulator.

[0108] Cell surface expression of TfR was analyzed by FACS (Figs. 1d-1k). The data show that the receptor is widespread and highly expressed on the surface of various human tumors. Thus, any of the human tumor cell lines can be used as a target for testing therapeutic utilities that are based on inhibition of iron uptake by TfR. The expression of HFE and TfR genes in resting versus activated human lymphocytes was tested (data not shown). These assays demonstrated that the expression of TfR is induced dramatically following activation, as reported previously (Bayer, 1999, Drobyski, 1996). Hence, lymphocyte activation depends on iron supply and TfR expression. Consequently, interference with iron uptake may lead to lymphocyte inactivation. This system may serve as a model for inactivation of autoimmune lymphocytes.

Sequence Analysis of Plasmid Encoding a Soluble β2m/HFE Monochain

[0109] Using recombinant DNA techniques a recombinant DNA fragment was engineered containing β2m and the α1-α3 region of human HFE that are linked via a DNA linker (described in "Specific Techniques"). The individual fragments were amplified by PCR and cloned into expression vectors harboring his-tag. DNA and amino acid sequences are presented (Fig. 2).

HHFE-Transfected Cells and β2m/HFE Monochain Transfected Cells Express and Secret the Encoded Molecules, Respectively

[0110] Human embryonal kidney cells transfected with HFE and derivatives (HFE, mutHFE, β2m/HFE/Mh and β2m/HFE/Mhm) were selected and screened for the expression of the transfected gene by RT-PCR using the relevant primers. Positive clones

expressing the transfected genes were chosen for further analysis. 293/HFE and 293/mutHFE clones express the encoded protein as determined by Western blot analysis of lysates from these clones, using antibodies directed against HFE-cytoplasmic tail (HFE-CT, data not shown). Cell extracts and conditioned media from clones positive for β2m/HFE/Mhm were tested by Western blot analysis for the expression of the encoded protein using anti-myc antibodies (9E10 hybridoma) (Fig. 3). The results show that the β2m/HFE/Mhm protein is expressed and is being secreted to the medium. The monochain is stable in 10% serum for at least 24 hours.

The β2m/HFE Monochain Binds to TfR

[0111] The association of human HFE and β2m/HFE monochain with TfR was tested. Following metabolic labeling and immunoprecipitation of representative clones with anti-TfR (V1-10) and anti-hβ2m (B2.62.2) monoclonal antibodies, the expression of β2m/HFE monochain or HFE that are not associated with TfR was detected with anti-HFE antibody, 2F5. The latter recognize an epitope on β2m/HFE complexes which is masked following binding to TfR (Vahdati et al, submitted to publication). Fig. 4 summarizes the data for HEK293 cells, 293/HFE, 293/β2m/HFE/Mh and 293/β2m/HFE/Mhm. The data demonstrate that the parental 293 cells express TfR, β2m (12 kD, mostly associated with class I heavy chains (45 kD)), and do not express any detectable level of endogenous HFE. 293/HFE cells express HFE/β2m heterodimers (as detected by 2F5) and TfR-associated HFE/β2m complexes, as detected mainly by anti-β2m (B2.62.2) and to much lower extent by anti-TfR (V1-10). 293/β2m/HFE/Mh cells express the expected monochain protein (55 kD) as indicated by its co-precipitation with anti-TfR (V1-10), but both HFE and β2m epitopes were not recognized by the respective mAbs, suggesting that the recombinant protein is not properly folded. 293/β2m/HFE/Mhm cells express the expected protein (55 kD). The protein associated with TfR as indicated

by its co-immunoprecipitation with anti-TfR mAb (V1-10), and is properly folded since it was recognized by anti-HFE (2F5) and anti-β2m (B2.62.2) mAbs. The fact that the association of the β2m/HFE/Mh protein was similar to that of the β2m/HFE/Mhm, implies that binding of HFE proteins to the TfR requires a linear sequence element that is independent of conformation. The following conclusions can be drawn from these data:

A. Proper folding of β2m/HFE monochains is influenced by the location of the His-tag.

B. In cells, β2m/HFE monochains are able to bind TfR independent of their conformation and as efficient as the wild type w.t. HFE.

Synthesis and Intracellular Trafficking of the β2m/HFE Monochain, HFE and TfR Molecules

[0112] HEK293, HEK293/HFE and HEK293/β2m/HFE/Mhm cells were metabolically labeled for 30 min, chased, lysed in NP40 containing buffer and immunoprecipitated with the relevant mAbs. Immunoprecipitates were fractionated on SDS-PAGE (Figs. 5a and 5b). The data in Fig. 5a show that the intracellular half-life of the β2m/HFE/Mhm molecule is ~8 hours. The β2m/HFE monochains co-immunoprecipitate with TfR (see co-immunoprecipitation with anti-TfR mAbs, V1-10). However, the half-life of the TfR-associated molecules is ~2 hours, indicating that these complexes dissociate readily. In the supernatant β2m/HFE/Mhm is evident already at 1 hour, and it appears to be stable and accumulates during the following 24 hours.

[0113] Fig. 5b shows that TfR-associated β2m/HFE complexes have a longer half-life (more than 24 hours).

[0114] The observation that the HFE signal in the TfR associated complexes increased during the first 4 hour chase, while the TfR signal decreased, suggesting that newly synthesized TfR associates first with "cold" HFE and, at a

later time, points surface TfR binds labeled β2m/HFE complexes at the cell surface.

[0115] The compiled data demonstrate that:

A. Most of the β2m/HFE/Mhm molecules are transported rapidly through the secretory pathway and are secreted to the media. Some of these molecules associate with TfR but have a relatively short half-life.

B. β2m/HFE complexes might have the capacity to associate with TfR on the cell surface.

C. β2m/HFE/Mhm molecules are stable in the supernatant for at least 24 hours.

HFE and β2m/HFE Monochain Cannot Be Over-Expressed in Some Human Tumor Cell Lines

[0116] The ability of HFE to inhibit iron uptake and reduce intracellular iron pools raised the possibility that over-expression of HFE may cause growth arrest of tumor cells that utilize the TfR as the main iron uptake system. To test this possibility directly and to determine whether the β2m/HFE monochain can exert the same effect, HeLa cells were transfected with wild type HFE, mutC282Y/HFE and β2m/HFE/Mhm expression vectors.

[0117] G418 resistant clones were selected and screened by RT-PCR and immunoprecipitations for the expression of the transfected genes. The total number of individual G418 resistant clones in each transfection was the same. The results of RT-PCR on mixed populations from each transfection are shown in Fig. 6a. The data demonstrate that mixed population from each transfection express comparable levels of the transfected gene. However, when tested for protein expression by immunoprecipitation analysis with the relevant antibodies only the monochain, but not the wild type HFE, could be detected (Fig. 6b, immunoprecipitation with anti-TfR, V1-10 and with anti HFE, 2F5). Moreover, analysis of individual

clones revealed that in monochain transfected clones that are G418 resistant, only 30% express the β2m/HFE monochain as determined by immunoprecipitation, data not shown. Therefore, the expression of HFE and β2m/HFE monochain appears to be down-regulated at the post-transcriptional level in HeLa cells. A similar phenomenon was observed in a prostate cancer cell line (PC3) transfected with the HFE gene, data not shown. It appears that the wild-type HFE, and to a lesser extent the β2m/HFE monochain, are subjected to negative regulation in certain human tumor cells (in HeLa and PC3 but not in HEK293 cells). The data suggest that the expression of the HFE and HFE derivatives interferes with cell growth and/or survival.

Radiolabeled β2m/HFE Monochain Binds to TfR on the Surface of Target Cells

[0118] HEK293/β2m/HFE/Mhm cells were metabolically labeled for 4 hours, washed and incubated in DCCM-1 for overnight. The media enriched in radiolabeled monochain was collected and further incubated with mouse and human cultured tumor cell lines. Binding to TfR was examined following immunoprecipitation of TfR-associated HFE complexes from cell lysates at 1 and 24 hours (Figs. 7a and 7b). Fig. 7a shows that the β2m/HFE monochain co-immunoprecipitated with TfR expressed by target cells. In the human cell lines (FO1 and HeLa), β2m/HFE monochain signal was observed following 1 hour incubation with target cells and was increased at 24 hours. In mouse cells (VAD12.79, B16) a signal was observed following 1 hour of incubation, but no signal was evident at 24 hours. Interestingly, in VAD12.79/hTfR cells, accumulation of β2m/HFE complexes could be detected at 24 hours. The binding of human HFE to mouse TfR is of very low affinity (Vahdati et al, submitted) supporting the fact that such complexes may penetrate cells inefficiently. Identical data are shown in Fig. 7b. This figure also demonstrates that β2m/HFE complexes (not associated with TfR, as indicated by their

immunoprecipitation with 2F5) accumulated in human cells. The latter phenomenon could result from dissociation of TfR-associated complexes inside the cells. The different accumulation pattern of β2m/HFE monochain in mouse and human cells suggests that the trafficking pathway of the β2m/HFE monochain in cells is dictated by its binding to human TfR. The data in these experiments substantiate the ability of the monochain to target cells specifically via the TfR, and to accumulate in cells for a relatively long period of time.

Purified β2m/HFE Monochain Penetrates the Cells and Co-Localizes with Transferrin

[0119] β2m/HFE monochain was purified from HEK293/β2m/HFE/Mhm conditioned media as described in the "Specific Techniques" section. The 55 kD monochain was clearly detected by Coomassie blue staining (Fig. 8, upper panel) and Western blot analysis using anti-myc antibodies (Fig. 8, lower panel). The purified β2m/HFE was tested for its potential to penetrate cells. VAD12.79 cells grown on cover glass slides were incubated with the monochain, rhodamine-labeled-transferrin or both. The cells were fixed and stained with anti-human β2m antibodies and a fluorescent-labeled secondary relevant antibody. Analysis was performed by confocal microscopy (results not shown). The results show that the monochain is localized to vesicular punctuated structures in the cytoplasm of the cells, suggesting that it was internalized into endocytic vesicles, like transferrin (Fig. 9a). Moreover, it is evident that at least part of the monochains is co-localized to the same vesicular structures (probably endosomes) as the transferrin.

Monochains Coupled to a Fluorescent Chemical Penetrate Target Cells

[0120] Purified monochains were chemically linked to FITC (fluorescein isothiocyanate), as described in "specific techniques". VAD12.79 cells grown on slides were incubated with the FITC-conjugated monochain, transferrin-conjugated-

transferrin or both. The cells were fixed and analyzed by confocal microscopy (results not shown). The results showed that both transferrin and the monochain penetrated the cells, localized in vesicular structures and in fact, a large part of the two molecules was localized to the same organelles, probably, endosomes.

GFP/Monochain, Penetrates Human Cells

[0121] In order to follow the trafficking of β2m/HFE monochain in cells and its internalization by target cells and, in addition, to explore the option of conjugating additional proteins to the monochain for drug delivery applications, a GFP-monochain (β2m/HFE/Mg) was engineered. If a β2m/HFE/Mg monochain penetrates the cells it would imply that other proteins, such as toxins, regulatory proteins, and antibodies, could be targeted to the endosomes via the β2m/HFE platform. HeLa and HEK293 cells were transfected with the β2m/HFE/Mg expression vector. G418 resistant clones were selected and screened by FACS for the expression of β2m/HFE/Mg monochain (data not shown). While 90% of the HEK293 clones expressed β2m/HFE/Mg monochain, only 10% of the HeLa clones expressed the protein. Since the transfection efficiency of both cell lines was the same, these data further support the previous assumption that HFE-derivatives are down-regulated in HeLa cells.

[0122] Since HFE is a negative regulator of iron uptake it was reasoned that its function might be manifested in the presence of the TfR ligand, the transferrin. Therefore, the binding and internalization of β2m/HFE/Mg by target cells either in the absence or presence of human transferrin in the cultured media was tested. HeLa cells grown on cover glass slides were incubated with conditioned media enriched with β2m/HFE/Mg protein with or without human transferrin-conjugated-transferrin. The cells were fixed, washed in PBS and mounted (results not shown). The β2m/HFE/Mg protein did

not penetrate HeLa cells in media devoid of human transferrin. However, in the presence of human transferrin, the β2m/HFE/Mg was internalized by cells and was partially co-localized with transferrin, probably in the endosomes. The monochain level decreased dramatically following 2 hours incubation, while the transferrin level was still high. The data clearly demonstrated that a monochain constructed from three polypeptides and based on the β2m/HFE core, penetrates cells via a pathway that depends on TfR/transferrin interaction.

[0123] The following conclusions can be drawn from these results:

A. A complex molecule that consists of the soluble β2m/HFE and GFP is expressed, properly folded, transported via the secretory pathway to the surrounding media and functions as expected in mammalian cells. The target cells are able to uptake β2m/HFE/Mg for a limited period of time suggesting that the amount of transferrin, HFE or cell surface TfR limits the uptake.

B. The β2m/HFE/Mg is internalized by target cells only in the presence of human transferrin, suggesting that the binding of transferrin to TfR is essential for high affinity binding of β2m/HFE to TfR and/or that internalization of TfR/HFE complexes depends on transferrin-binding.

C. A portion of the β2m/HFE/Mg molecule is co-localized with transferrin, but for a limited period of time, suggesting that HFE may dissociate from TfR complex and translocate to another cellular organelle.

D. The half-life of the β2m/HFE/Mg in the cells is relatively short indicating that either it is

degraded or that its fluorescence emission diminishes with time.

Decreased Level of Cellular TfR in Cells Treated with β2m/HFE/Mhm

[0124] Over-expression of HFE results in reduction of iron pools that consequently leads to up-regulation in the level of TfR and down-regulation in the level of the Fe-storage protein, ferritin (published data and data not shown). A study was designed to determine whether external addition of β2m/HFE monochain leads to the same results. HeLa cells were treated with purified monochains for 24 hours and TfR levels were monitored by Western blot analysis. Fig. 9 shows that treatment of HeLa cells with β2m/HFE, reduced by at least 50% the level of TfR. Treatment with a Fe-chelating agent (DFO) had a marginal effect on the levels of TfR. The decrease was specific to TfR since such a decrease was not observed in the level of other proteins like MAP kinase. These data, in addition to the data shown in the previous paragraph (block in monochain uptake following 2 hours incubation with the monochain), suggest that β2m/HFE/Mg induces intracellular degradation of TfR.

[0125] From all of these experiments, the following conclusions emerge:

A. A recombinant DNA fragment encodes a soluble β2m/HFE monochain in mammalian cells. The β2m/HFE monochain fold depends on the location of the His-tag in the vector. A β2m/HFE/Mhm is properly folded, soluble, stable and rapidly secreted to the supernatant from transfected cells.

B. β2m/HFE/Mhm binds to TfR intracellularly independent of its conformation.

C. β2m/HFE/Mhm binds to TfR on the surface of tumor cells.

D. β2m/HFE/Mhm penetrates tumor cells via a TfR-dependent pathway.

E. β2m/HFE/Mhm can be chemically coupled to other chemicals (for example, FITC) or synthesized as a more complex, tri-partner protein (for example, β2m/HFE/Mg) and keep its characteristics. Thus, the latter protein conjugates bind and penetrate cells in a TfR-dependent pathway.

F. Some tumor cell lines are sensitive to high levels of HFE and HFE derivatives (HeLa, PC3), suggesting that HFE derivatives can interfere with growth.

G. Externally added β2m/HFE/Mhm reduces the level of TfR in some tumor cell lines and thus interferes with iron metabolism.

[0126] The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed functions may take a variety of alternative forms without departing from the invention. Thus the expressions "means to..." and "means for...", or any method step language, as may be found in the specification above and/or in the claims below, followed by a functional statement, are intended to define and cover whatever structural, physical, chemical or electrical element or structure, or whatever method

step, which may now or in the future exist which carries out the recited function, whether or not precisely equivalent to the embodiment or embodiments disclosed in the specification above, i.e., other means or steps for carrying out the same functions can be used; and it is intended that such expressions be given their broadest interpretation.

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Zhou et al, "HFE gene knockout produces mouse model of hereditary hemochromatosis", Proc Natl Acad Sci USA 95(5):2492-2497 (1998)

WHAT IS CLAIMED IS:

1. A soluble β2m/HFE monochain which is a polypeptide comprising human β2m, or an analog or active fragment thereof, linked to the α1-α3 domains of human HFE, or an analog or active fragment thereof, by means of a flexible linker peptide, or a functional derivative or salt of said polypeptide.

2. A monochain in accordance with claim 1, further including a therapeutic bound thereto.

3. A DNA molecule comprising a nucleotide sequence encoding the monochain of claim 1 or claim 2.

4. A vector containing the DNA of claim 3

5. A cell transformed with a vector in accordance with claim 4.

6. A method of producing a monochain in accordance with claim 1 or claim 2, comprising culturing cells in accordance with claim 5 under conditions such that said monochain is expressed and collecting the monochain so expressed.

7. A method for reducing intracellular iron absorption, comprising administering to a patient in need of reduction of intracellular iron absorption an effective amount of a monochain in accordance with claim 1.

8. A method for reducing intracellular iron absorption, comprising administering to a patient in need of reduction of intracellular iron absorption an effective amount of β2m or an analog, active fragment, functional derivative or salt thereof.

9. A method for reducing intracellular iron absorption, comprising administering to a patient in need of reduction of intracellular iron absorption an effective amount of HFE or an analog, active fragment, functional derivative or salt thereof.

10. A method for delivering a therapeutic to cells that over-express the transferrin receptor (TfR), comprising administering a monochain in accordance with claim 2.

11. A method for delivering a therapeutic across the blood-brain barrier, comprising administering a monochain in accordance with claim 2.

12. A monochain in accordance with claim 1 or claim 2, wherein said linker is one to five repetitions of GGGGS (SEQ ID NO:15).

13. A monochain in accordance with any one of claims 1, 2, or 12, further including a protease cleavage site in or adjacent to said linker.

14. A monochain in accordance with claim 2, further including a protease cleavage site between said therapeutic and the rest of said monochain.

15. A monochain in accordance with claim 2, wherein said therapeutic is a chemical, protein, peptide, DNA or oligonucleotide conjugated directly to the monochain or indirectly via liposomes or polymer-modified liposomes.

16. A method for treatment of a brain tumor comprising administering a monochain in accordance with any one of claims 2, 14 or 15, wherein said therapeutic is one which is effective against said brain tumor, whereby said monochain will deliver said therapeutic across the blood-brain barrier.

17. A method in accordance with claim 10, wherein said cells are activated lymphocytes or leukocytes.

18. A method in accordance with claim 9, wherein said patient in need of reduction of iron absorption is one having hereditary hemochromatosis, transfusions, thalassemias, hemolytic anemia or chronic infections.

19. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a monochain in accordance with any one of claims 1, 2, or 12-15.

Fig. 1a

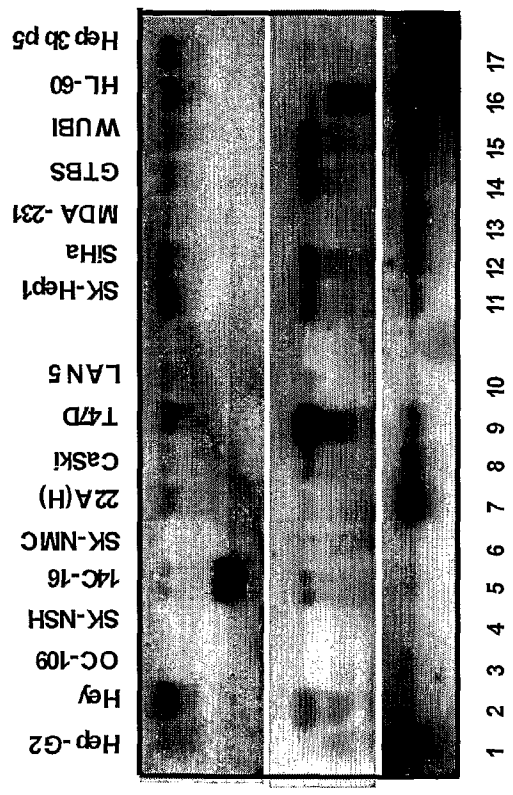
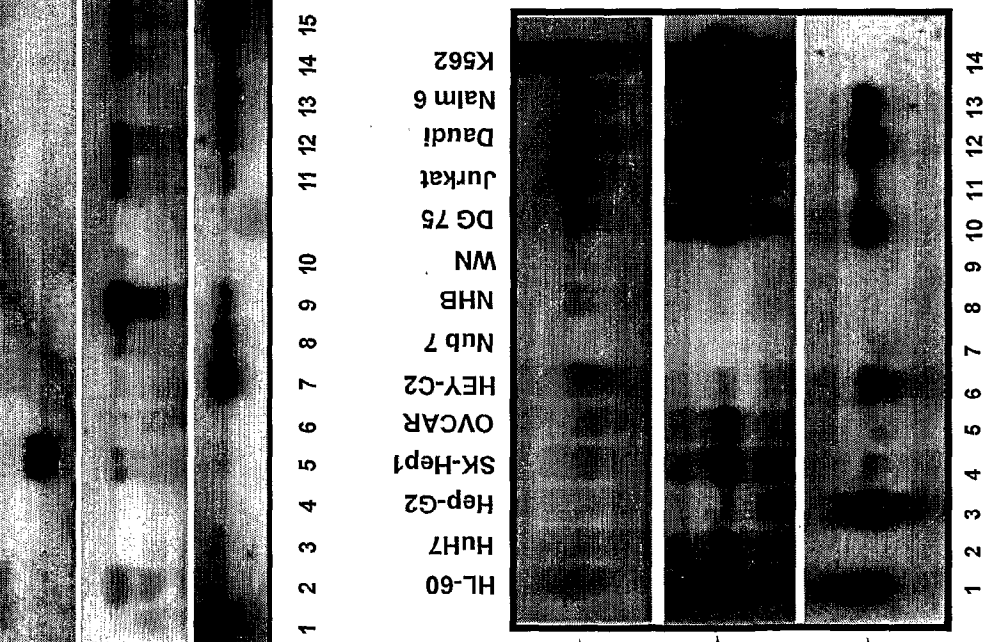


Fig. 1b



Cell Line		HFE	TfR	MHC Class I
Neuroblastoma	SK-NSH	-	-	-/low
	SK-NMC	-	-/low	-/low
	LAN 5	+	+	-/low
	Nub 7	-	-/low	+
	NHB	+	+	-/low
	WN	+	+	-/low
Hepatoma	Hep-G2	+	+	++
	SK-Hep1	++	++	+
	Hep 3b p5	++	+	++
	HuH7	+	++	-/low
Cervical Squamous Carcinoma	SiHa	++	++	++
Cervical Epidermal Carcinoma	CaSki	+	+	++
Melanoma	GTBS	++	++	++
	WUBI	++	++	+++
Ovarian Carcinoma	HEY	+++	+	+
	HEY C2	++	+	+++
	OVCAR	++	++	+
	OC109	-	-/low	+
Breast Carcinoma	T47D	++	+++	+
	MDA 231	+	+/low	+++
Squamous Cell Carcinoma	22A	+	+	+++
	14C-16		+	+
Burkitt's Lymphoma	Daudi	+++	+++	+++
	DG-75	+++	+++	+++
Pre B	Nalm 6	+++	+++	+++
T Cell Leukemia	Jurkat	+++	+++	++
Chronic Myelogonous Leukemia	K562	+++	+++	-/low
Promyelocytic Leukemia	HL-60	+++	+++	+++

Fig. 1c

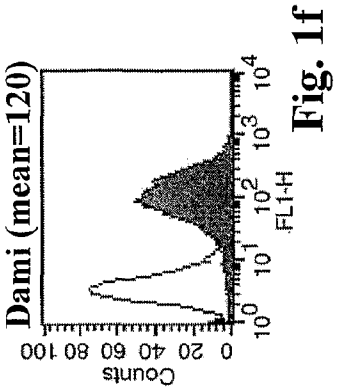
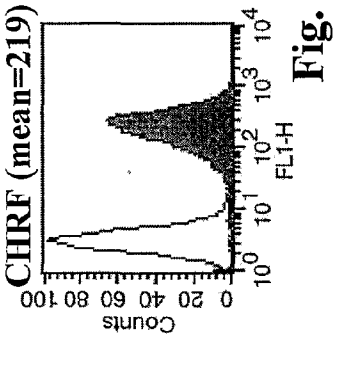


Fig. 1f



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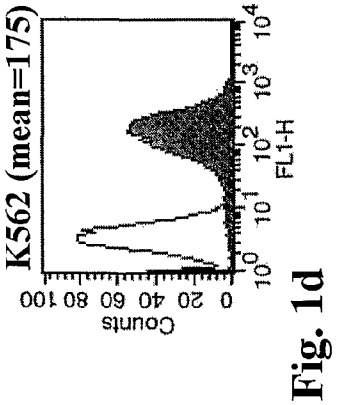

Fig. 1d

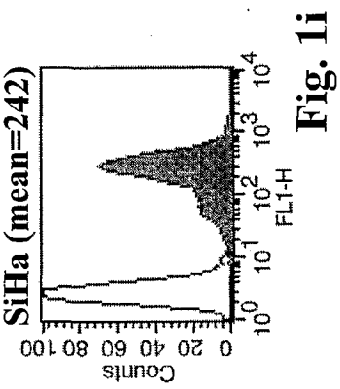

Fig. 1i

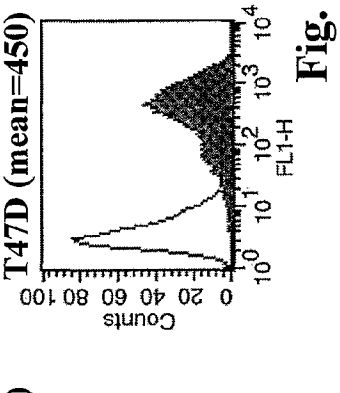

Fig. 1h

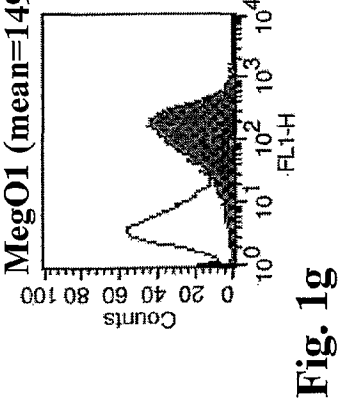

Fig. 1g

Fig. 1k

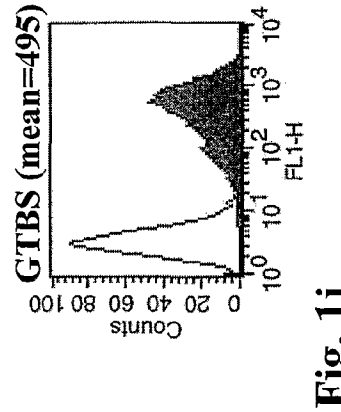

Fig. 1j

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met ser arg ser val ala leu ala val leu ala leu leu ser leu ser gly leu glu ala
61/21                                               91/31
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ile gln arg thr pro lys ile gln val tyr ser arg his pro ala glu asn gly lys ser
121/41                                              151/51
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asn phe leu asn cys tyr val ser gly phe his pro ser asp ile glu val asp leu leu
181/61                                              211/71
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lys asn gly glu arg ile glu lys val glu his ser asp leu ser phe ser lys asp trp
241/81                                              271/91
tct ttc tat ctc ttg tac tac act gaa ttc acc ccc act gaa aaa gat gag tat gcc tgc
ser phe tyr leu leu tyr tyr thr glu phe thr pro thr glu lys asp glu tyr ala cys
301/101                                             331/111
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arg val asn his val thr leu ser gln pro lys ile val lys trp asp arg asp met gly
361/121                                             391/131
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gly gly gly ser gly gly gly gly ser gly gly gly gly ser arg leu leu arg ser his
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ser leu his tyr leu phe met gly ala ser glu gln asp leu gly leu ser leu phe glu
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ala leu gly tyr val asp asp gln leu phe val phe tyr asp his glu ser arg arg val
541/181                                             571/191
gag ccc cga act cca tgg gtt tcc agt aga att tca agc cag atg tgg ctg cag ctg agt
glu pro arg thr pro trp val ser ser arg ile ser ser gln met trp leu gln leu ser
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gln ser leu lys gly trp asp his met phe thr val asp phe trp thr ile met glu asn
661/221                                             691/231
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his asn his ser lys glu ser his thr leu gln val ile leu gly cys glu met gln glu
721/241                                             751/251
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asp asn ser thr glu gly tyr trp lys tyr gly tyr asp gly gln asp his leu glu phe
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cys pro asp thr leu asp trp arg ala ala glu pro arg ala trp pro thr lys leu glu
841/281                                             871/291
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asn tyr tyr pro gln asn ile thr met lys trp leu lys asp lys gln pro met asp ala
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Fig. 2

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1141/381                                    1171/391
acc ttg gct gta ccc cct ggg gaa gag cag aga tat acg tgc cag gtg gag cac cca ggc
thr leu ala val pro pro gly glu glu gln arg tyr thr cys gln val glu his pro gly
1201/401                                    1231/411
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leu asp gln pro leu ile val ile trp gly leu glu ser arg gly pro phe glu gln lys
1261/421                                    1291/431
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Fig. 2 (continued)

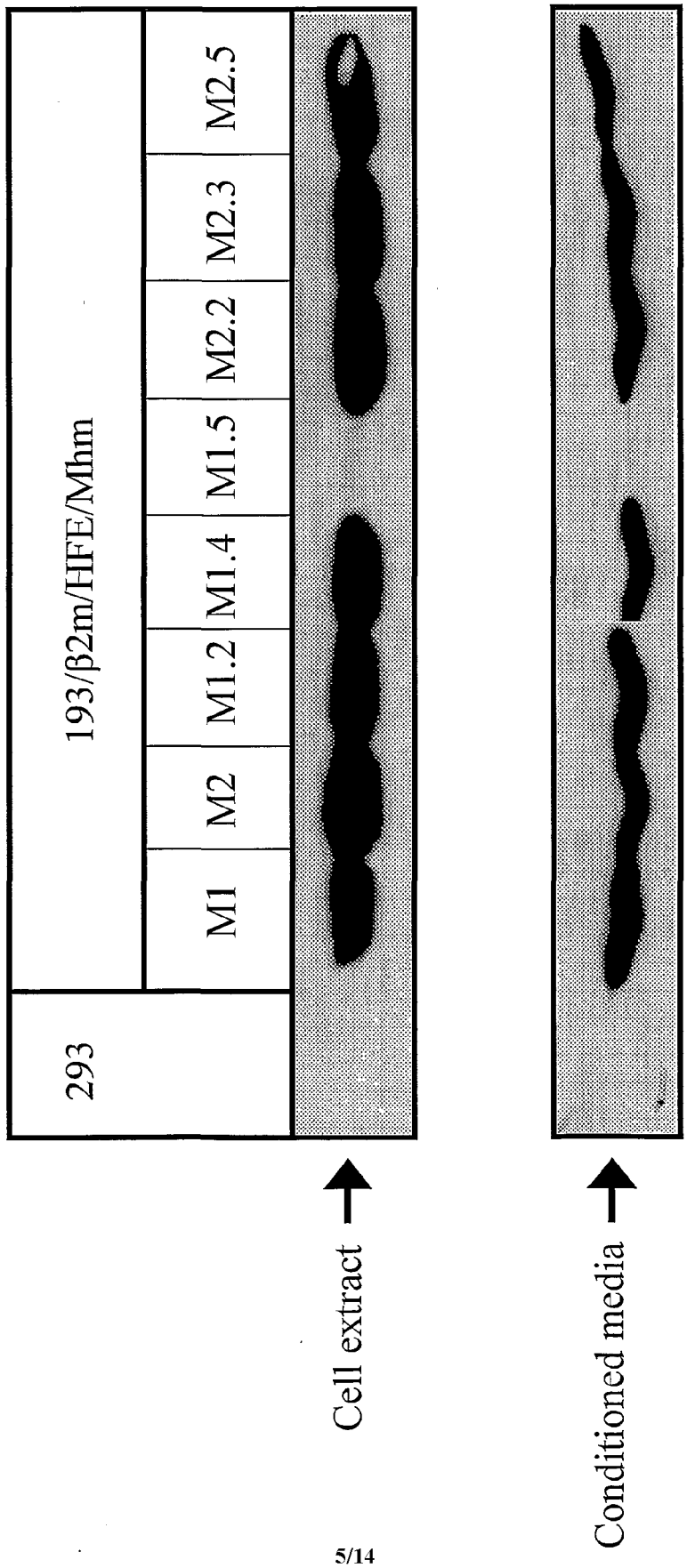
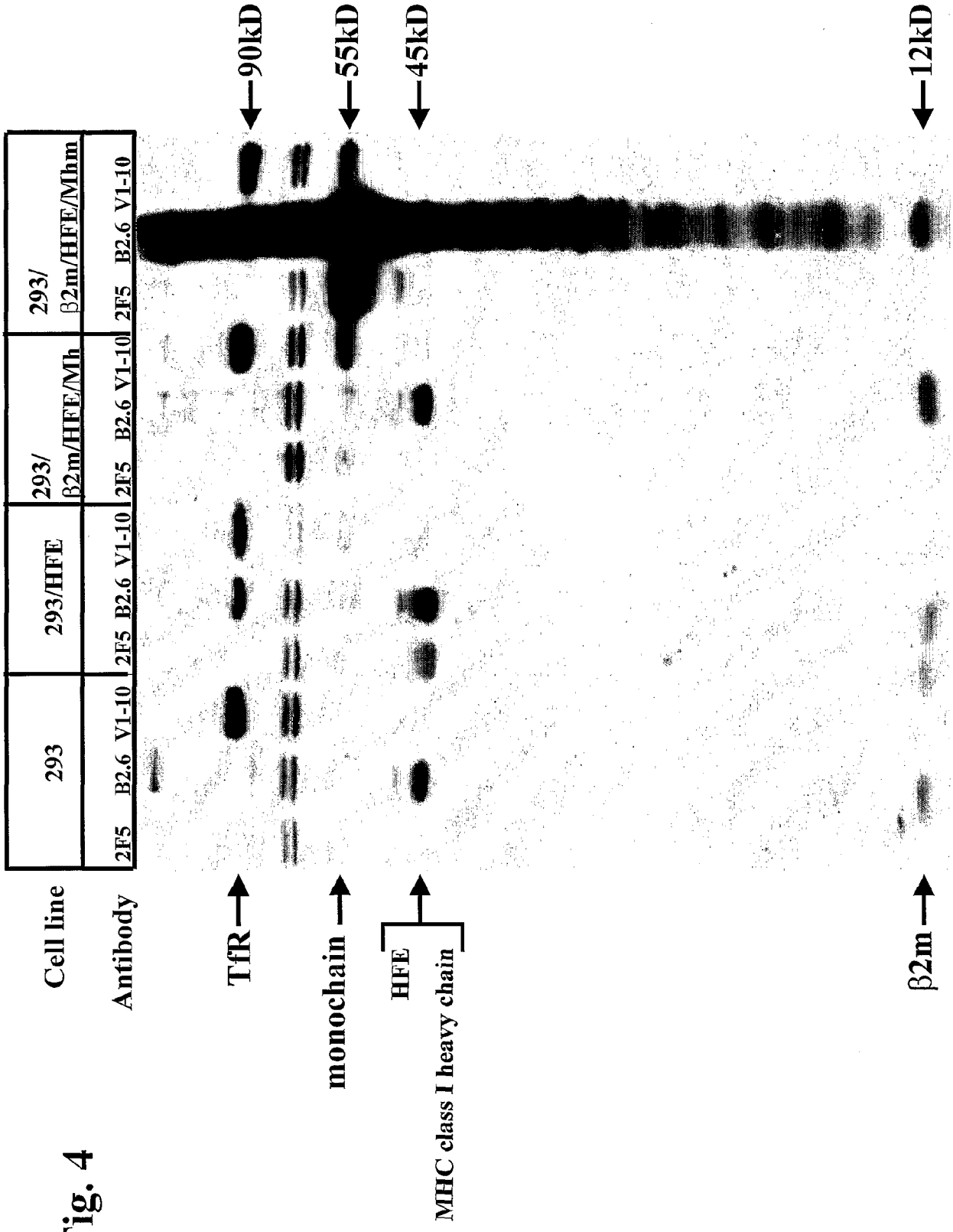
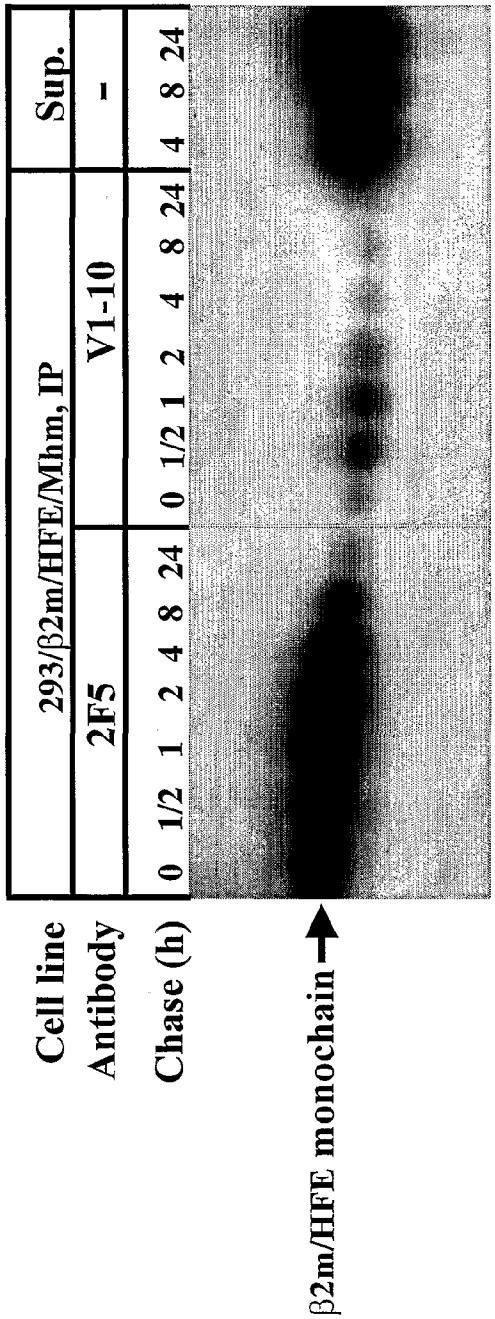

293
193/β2m/HFE/Mhm
M1
M2
M1.2
M1.4
M1.5
M2.2
M2.3
M2.5
Cell extract
Conditioned media

Fig. 3

Fig. 4


Cell line
293
293/HFE
293/ β2m/HFE/Mh
293/ β2m/HFE/Mhm
Antibody
2F5 B2.6 V1-10 2F5 B2.6 V1-10 2F5 B2.6 V1-10 2F5 B2.6 V1-10
90kD
55kD
45kD
12kD
TfR
monochain
HFE
MHC class I heavy chain
β2m


Cell line
293/β2m/HFE/Mhm, IP
Sup.
Antibody
2F5
V1-10
-
Chase (h)
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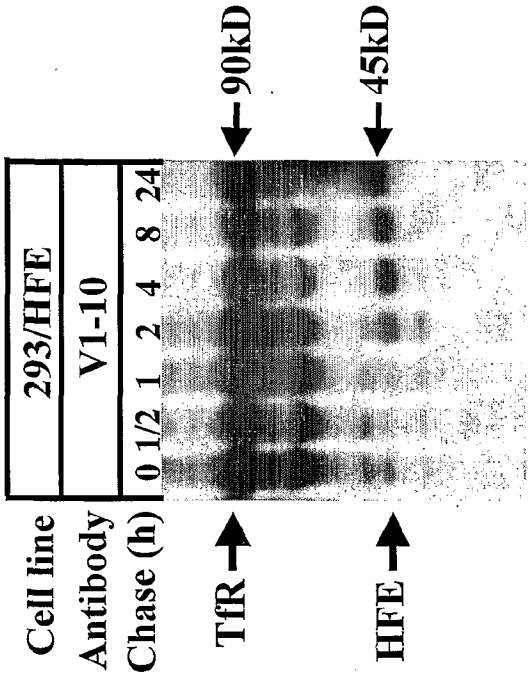
0 1/2 1 2 4 8 24
4 8 24
β2m/HFE monochain

Fig. 5a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Antibody
V1-10
Chase (h)
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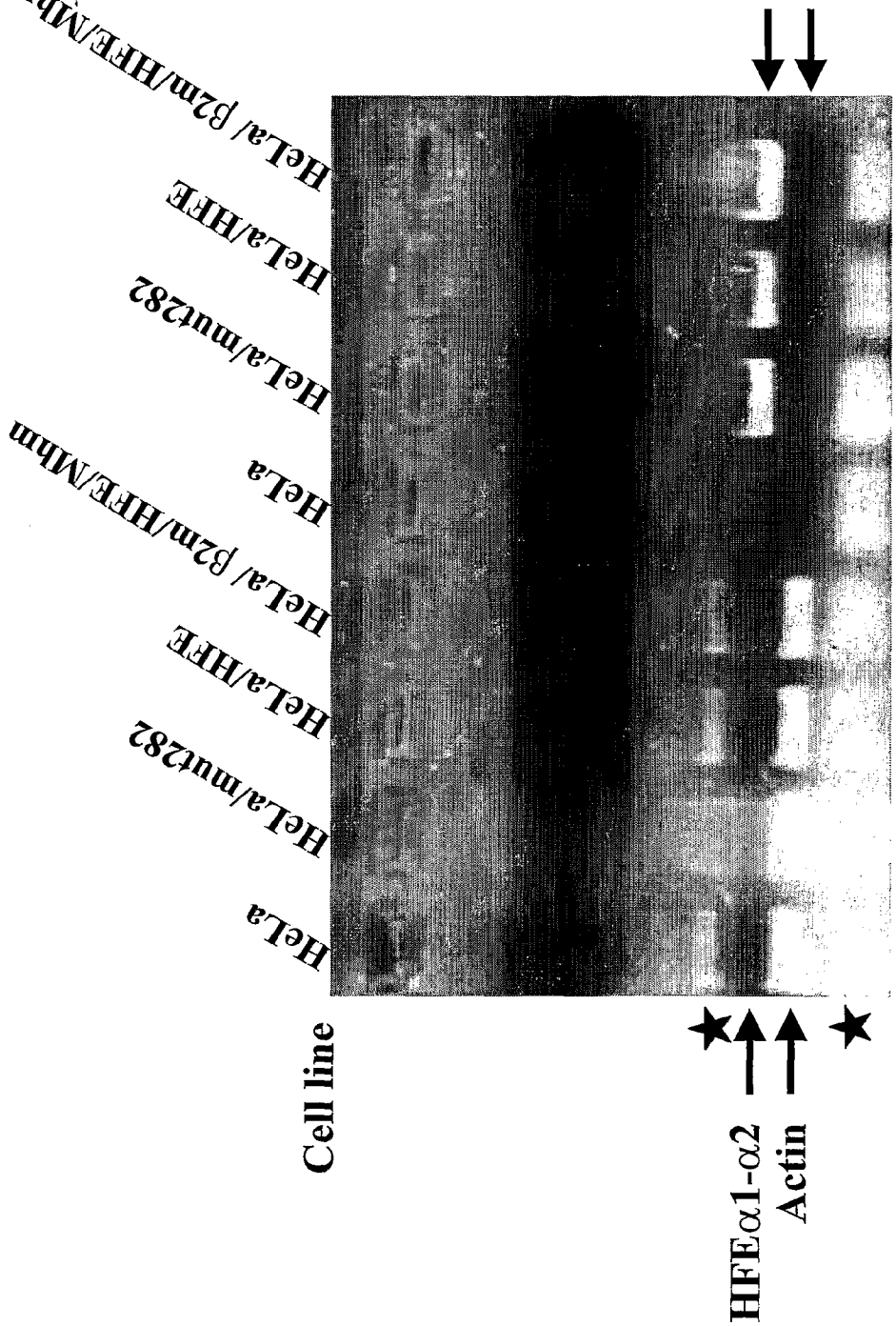
90kD
45kD
TfR
HFE

Fig. 5b


HeLa
HeLa/mut282
HeLa/HFE
HeLa/ β2m/HFE/Mhm
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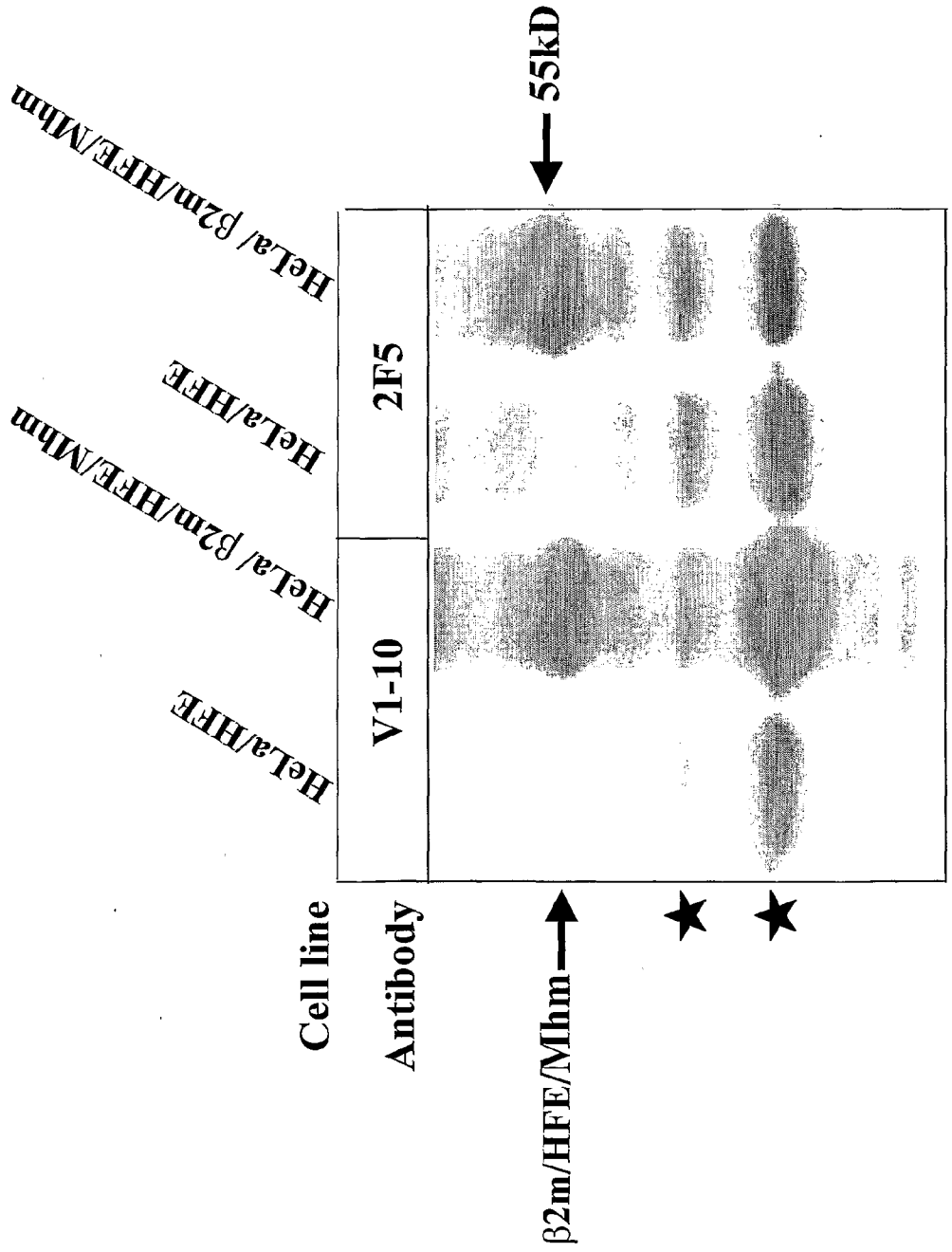
HeLa/ β2m/HFE/Mhm
Cell line
HFEα1-α2
Actin

Fig. 6a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HeLa/HFE
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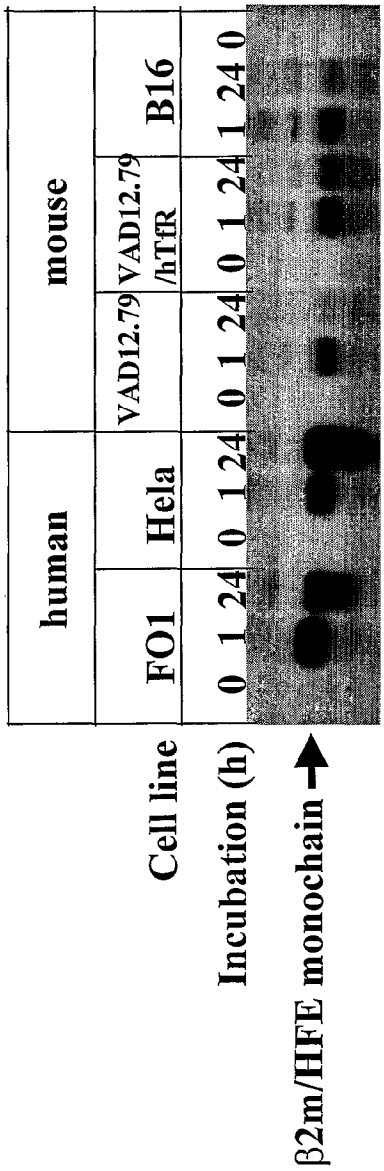
V1-10
Cell line
Antibody
β2m/HFE/Mhm

Fig. 6b


human
mouse
FO1
Hela
VAD12.79
VAD12.79 /hTfR
B16
Cell line
Incubation (h)
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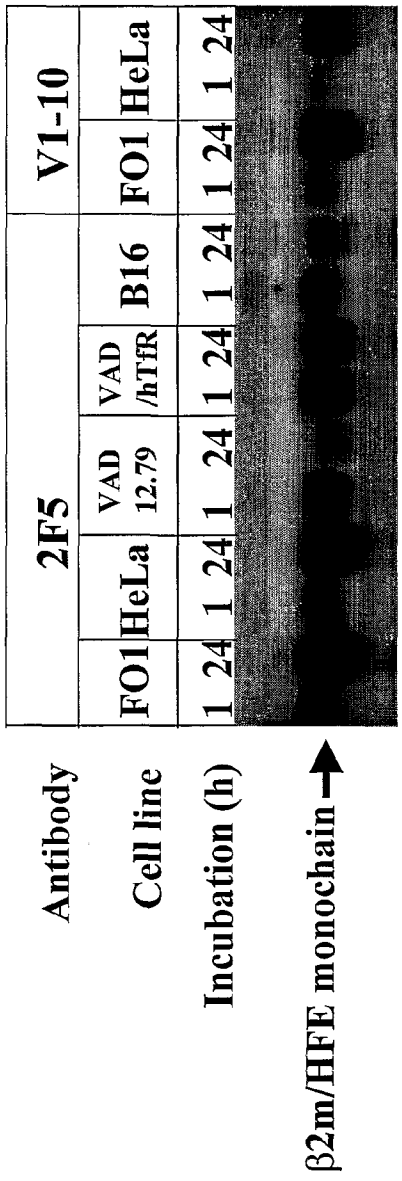
β2m/HFE monochain

Fig. 7a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2F5
V1-10
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FO1
HeLa
VAD 12.79
VAD /hTfR
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HeLa
Incubation (h)
1 24 1 24 1 24 1 24 1 24 1 24 1 24
β2m/HFE monochain

Fig. 7b

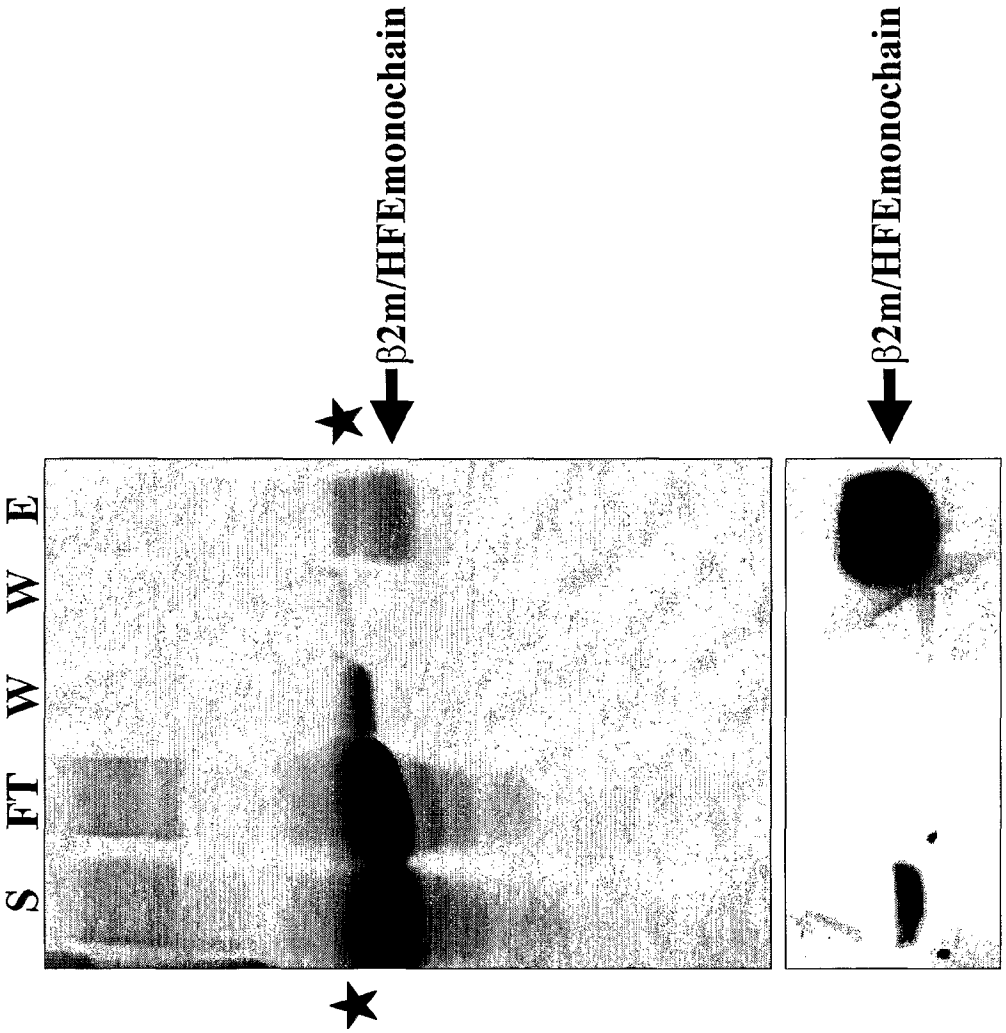

Fig. 8

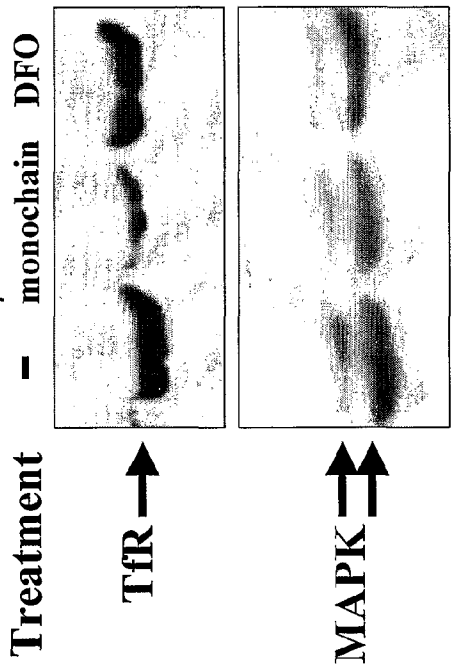

Fig. 9

SEQUENCE LISTING

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ROTEM-YEHUDAR, Rimat
LAHAM, Nihay

<120> A SOLUBLE BETA 2 MICROGLOBULIN (ß2M)/HFE MONOCHAIN FOR BIOTECHNOLOGIC AL AND
THERAPEUTIC APPLICATIONS

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Gly Leu Glu Ala Ile Gln Arg Thr Pro Lys Ile Gln Val Tyr Ser Arg
            20                  25                  30

cat cca gca gag aat gga aag tca aat ttc ctg aat tgc tat gtg tct      144
His Pro Ala Glu Asn Gly Lys Ser Asn Phe Leu Asn Cys Tyr Val Ser
        35                  40                  45

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Gly Phe His Pro Ser Asp Ile Glu Val Asp Leu Leu Lys Asn Gly Glu
    50                  55                  60

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Arg Ile Glu Lys Val Glu His Ser Asp Leu Ser Phe Ser Lys Asp Trp
65                  70                  75                  80

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Ser Phe Tyr Leu Leu Tyr Tyr Thr Glu Phe Thr Pro Thr Glu Lys Asp
                85                  90                  95

gag tat gcc tgc cgt gtg aac cat gtg act ttg tca cag ccc aag ata      336
Glu Tyr Ala Cys Arg Val Asn His Val Thr Leu Ser Gln Pro Lys Ile
            100                 105                 110
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        115                 120                 125

tcc ggt ggc ggc ggt tcc cgc ttg ctg cgt tca cac tct ctg cac tac      432
Ser Gly Gly Gly Gly Ser Arg Leu Leu Arg Ser His Ser Leu His Tyr
    130                 135                 140

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Leu Phe Met Gly Ala Ser Glu Gln Asp Leu Gly Leu Ser Leu Phe Glu
145                 150                 155                 160

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Ala Leu Gly Tyr Val Asp Asp Gln Leu Phe Val Phe Tyr Asp His Glu
                165                 170                 175

agt cgc cgt gtg gag ccc cga act cca tgg gtt tcc agt aga att tca      576
Ser Arg Arg Val Glu Pro Arg Thr Pro Trp Val Ser Ser Arg Ile Ser
            180                 185                 190

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        195                 200                 205

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Met Phe Thr Val Asp Phe Trp Thr Ile Met Glu Asn His Asn His Ser
    210                 215                 220

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225                 230                 235                 240

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Asp Asn Ser Thr Glu Gly Tyr Trp Lys Tyr Gly Tyr Asp Gly Gln Asp
                245                 250                 255

cac ctt gaa ttc tgc cct gac aca ctg gat tgg aga gca gca gaa ccc      816
His Leu Glu Phe Cys Pro Asp Thr Leu Asp Trp Arg Ala Ala Glu Pro
            260                 265                 270

agg gcc tgg ccc acc aag ctg gag tgg gaa agg cac aag att cgg gcc      864
Arg Ala Trp Pro Thr Lys Leu Glu Trp Glu Arg His Lys Ile Arg Ala
        275                 280                 285

agg cag aac agg gcc tac ctg gag agg gac tgc cct gca cag ctg cag      912
Arg Gln Asn Arg Ala Tyr Leu Glu Arg Asp Cys Pro Ala Gln Leu Gln
    290                 295                 300

cag ttg ctg gag ctg ggg aga ggt gtt ttg gac caa caa gtg cct cct      960
Gln Leu Leu Glu Leu Gly Arg Gly Val Leu Asp Gln Gln Val Pro Pro
305                 310                 315                 320

ttg gtg aag gtg aca cat cat gtg acc tct tca gtg acc act cta cgg     1008
Leu Val Lys Val Thr His His Val Thr Ser Ser Val Thr Thr Leu Arg
                325                 330                 335

tgt cgg gcc ttg aac tac tac ccc cag aac atc acc atg aag tgg ctg     1056
Cys Arg Ala Leu Asn Tyr Tyr Pro Gln Asn Ile Thr Met Lys Trp Leu
            340                 345                 350
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aag gat aag cag cca atg gat gcc aag gag ttc gaa cct aaa gac gta     1104
Lys Asp Lys Gln Pro Met Asp Ala Lys Glu Phe Glu Pro Lys Asp Val
        355                 360                 365

ttg ccc aat ggg gat ggg acc tac cag ggc tgg ata acc ttg gct gta     1152
Leu Pro Asn Gly Asp Gly Thr Tyr Gln Gly Trp Ile Thr Leu Ala Val
    370                 375                 380

ccc cct ggg gaa gag cag aga tat acg tgc cag gtg gag cac cca ggc     1200
Pro Pro Gly Glu Glu Gln Arg Tyr Thr Cys Gln Val Glu His Pro Gly
385                 390                 395                 400

ctg gat cag ccc ctc att gtg atc tgg ggg ctc gag tct aga ggg ccc     1248
Leu Asp Gln Pro Leu Ile Val Ile Trp Gly Leu Glu Ser Arg Gly Pro
                405                 410                 415

ttc gaa caa aaa ctc atc tca gaa gag gat ctg aat atg cat acc ggt     1296
Phe Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Met His Thr Gly
            420                 425                 430

cat cat cac cat cac cat tga                                        1317
His His His His His His
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Met Ser Arg Ser Val Ala Leu Ala Val Leu Ala Leu Leu Ser Leu Ser
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Gly Leu Glu Ala Ile Gln Arg Thr Pro Lys Ile Gln Val Tyr Ser Arg
            20                  25                  30

His Pro Ala Glu Asn Gly Lys Ser Asn Phe Leu Asn Cys Tyr Val Ser
        35                  40                  45

Gly Phe His Pro Ser Asp Ile Glu Val Asp Leu Leu Lys Asn Gly Glu
    50                  55                  60

Arg Ile Glu Lys Val Glu His Ser Asp Leu Ser Phe Ser Lys Asp Trp
65                  70                  75                  80

Ser Phe Tyr Leu Leu Tyr Tyr Thr Glu Phe Thr Pro Thr Glu Lys Asp
                85                  90                  95

Glu Tyr Ala Cys Arg Val Asn His Val Thr Leu Ser Gln Pro Lys Ile
            100                 105                 110
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Val Lys Trp Asp Arg Asp Met Gly Gly Gly Gly Ser Gly Gly Gly Gly
        115                 120                 125

Ser Gly Gly Gly Gly Ser Arg Leu Leu Arg Ser His Ser Leu His Tyr
    130                 135                 140

Leu Phe Met Gly Ala Ser Glu Gln Asp Leu Gly Leu Ser Leu Phe Glu
145                 150                 155                 160

Ala Leu Gly Tyr Val Asp Asp Gln Leu Phe Val Phe Tyr Asp His Glu
                165                 170                 175

Ser Arg Arg Val Glu Pro Arg Thr Pro Trp Val Ser Ser Arg Ile Ser
            180                 185                 190

Ser Gln Met Trp Leu Gln Leu Ser Gln Ser Leu Lys Gly Trp Asp His
        195                 200                 205

Met Phe Thr Val Asp Phe Trp Thr Ile Met Glu Asn His Asn His Ser
    210                 215                 220

Lys Glu Ser His Thr Leu Gln Val Ile Leu Gly Cys Glu Met Gln Glu
225                 230                 235                 240

Asp Asn Ser Thr Glu Gly Tyr Trp Lys Tyr Gly Tyr Asp Gly Gln Asp
                245                 250                 255

His Leu Glu Phe Cys Pro Asp Thr Leu Asp Trp Arg Ala Ala Glu Pro
            260                 265                 270

Arg Ala Trp Pro Thr Lys Leu Glu Trp Glu Arg His Lys Ile Arg Ala
        275                 280                 285

Arg Gln Asn Arg Ala Tyr Leu Glu Arg Asp Cys Pro Ala Gln Leu Gln
    290                 295                 300

Gln Leu Leu Glu Leu Gly Arg Gly Val Leu Asp Gln Gln Val Pro Pro
305                 310                 315                 320

Leu Val Lys Val Thr His His Val Thr Ser Ser Val Thr Thr Leu Arg
                325                 330                 335

Cys Arg Ala Leu Asn Tyr Tyr Pro Gln Asn Ile Thr Met Lys Trp Leu
            340                 345                 350
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Lys Asp Lys Gln Pro Met Asp Ala Lys Glu Phe Glu Pro Lys Asp Val
        355                 360                 365

Leu Pro Asn Gly Asp Gly Thr Tyr Gln Gly Trp Ile Thr Leu Ala Val
    370                 375                 380

Pro Pro Gly Glu Glu Gln Arg Tyr Thr Cys Gln Val Glu His Pro Gly
385                 390                 395                 400

Leu Asp Gln Pro Leu Ile Val Ile Trp Gly Leu Glu Ser Arg Gly Pro
                405                 410                 415

Phe Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Met His Thr Gly
            420                 425                 430

His His His His His His
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Ala Glu Arg Glu
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1 5 10 15

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ccatctcgag gtcgccacca tggtgag 27

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